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(71) Applicant *(for all designated States except US)*: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: TSCHOPP, Jurg [CH/CH]; Chemin des Fontannins 10, CH–1066 Epalinges (CH).

(74) Agent: FLYNN, Kerry; Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: APRIL– A NOVEL PROTEIN WITH GROWTH EFFECTS

(57) Abstract

APRIL, a novel member of the tumor necrosis factor family (TNF), modified APRILs, and pharmaceutical compositions comprising them.

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Europäisches Patentamt
Eingangsstelle

European Patent Office
Receiving Section

Office européen des brevets
Section de Dépôt

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Datum/Date
05/07/00

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
JH/ml000789ep	98946066.2-2105 / 1027431

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Apotech R&D S.A.

NOTIFICATION OF EUROPEAN PUBLICATION NUMBER AND INFORMATION ON THE APPLICATION OF ARTICLE 67(3) EPC

The provisional protection under Article 67(1) and (2) EPC in the individual Contracting States becomes effective only when the conditions referred to in Article 67(3) EPC have been fulfilled (for further details, see information brochure of the European Patent Office "National Law relating to the EPC" and additional information in the Official Journal of the European Patent Office).

A request has been made for extension of the patent to
AL LT LV MK RO SI .
See Official Journal 1-2/1994 for further information on provisional protection in AL LT LV MK RO SI .

Pursuant to Article 158(1) EPC the publication under Article 21 PCT of an international application for which the European Patent Office is a designated Office takes the place of the publication of a European patent application.

The bibliographic data of the above-mentioned Euro-PCT application will be published on 16.08.00 in Section I.1 of the European Patent Bulletin.
The European publication number is 1027431.

In all future communications to the European Patent Office, please quote the application number plus Directorate number.

RECEIVING SECTION

(5009151) EPASYS (01.98) RECEIVING SECTION 28/06/00*2105
APPLICATION-NR: 98946066.2

WARNING : RPUB (EURO - PCT)

PACT : WO9912965/C07K14/00/18.03.99/9911/9922 FILL : EN
REFN : US9819191 DFIL : 11.09.98 TRAN :
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APPR : 01/03018010.3/DEST/
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INVT : 01(00.00.00)/1/TSCHOPP, Jurg/Chemin des Fontannins 10/CH-1066 Epalinges/CH

DEST : AT/01/05.04.00 BE/02/05.04.00 CH/03/05.04.00
LI/03/05.04.00 CY/04/05.04.00 DE/05/05.04.00
DK/06/05.04.00 ES/07/05.04.00 FI/08/05.04.00
FR/09/05.04.00 GB/10/05.04.00 GR/11/05.04.00
IE/12/05.04.00 IT/13/05.04.00 LU/14/05.04.00
MC/15/05.04.00 NL/16/05.04.00 PT/17/05.04.00
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EXAM : 05.04.00/05.04.00 CLMS : 013/05.04.00

IPCL : C12N15/11(14.04.00) OBSC : 01/C07K14/525
02/A61K38/00

TIDE : APRIL - EINE NEUE PROTEIN MIT WACHSTUMSWIRKUNGEN
TIEN : APRIL- A NOVEL PROTEIN WITH GROWTH EFFECTS(15.04.99)
TIFR : NOUVEAUX LIGANDS DE LA FAMILLE DES TNF(15.04.99)

DRAW : ///// BIOM : /00.00.00//

STATUS OF APPLICATION

FORA : 00.00.00//00.00.00/ ADWI : /00.00.00/00.00.00
WDRA : 00.00.00// REFU : /00.00.00/00.00.00

(5009151) EPASYS (01.98) RECEIVING SECTION 28/06/00*2105
APPLICATION-NR: 98946066.2

REMARKS : NONE
TEXT : NONE //

! ! ! ! PLAUSIBILITY ERRORS ! ! ! !
NO PLAUSIBILITY ERROR

Bibliographic and procedural data checked/corrected.
Publication file out, if publication TRAN (Art. 158(3) EPC).

RPUB 01 coded. ..... van der VELDEN..... Name/Date

Tel.: 31 70 3403691
28.06.00

Patentanwälte Patent Attorneys

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Our Ref.:
000789ep/JH/ml

Cologne,
May 19, 2000

European patent application 98946066.2-2105
"April-a novel protein with growth effects"
Apotech R&D S.A.

This is in reply to the Communication pursuant to Rules 109 and 110 EPC dated April 19, 2000.

Enclosed in triplicate is an amended set of claims (1-13) on which the present case shall be further prosecuted. Basis for the amended set of claims can be found as follows:

Claim 1: The subject matter of previous claims 1, 5, 9 and 21 have in whole or in part been included as alternatives in new claim 1. Support for section (a) relating to SEQ ID NO:4 can be found on page 6, line 11 of the specification. Support for section (b) relating to SEQ ID NO: 3 can be found on page 6, line 11 of the specification. Support for section (c) relating to 70% homology can be found on page 10, line 4 of the specification. Support for section (f) relating to amino acids 110 to 250 can be found on page 30, lins 6-7. Support for section (g) relating to amino acids 29 to 49 can be found in Fig. 1A which delineates the transmembrane region as 29-49.

Claim 2: corresponds to previous claims 6 and 7.

Claim 3 corresponds to previous claim 8.

Claim 4 corresponds to previous claim 10.

Claim 5 corresponds to previous claims 21 and 22.

Claim 6 corresponds to previous claim 12.

Claim 7 corresponds to previous claim 23.

Claim 8 corresponds to previous claim 24.

Claim 9 corresponds to previous claims 13, 15 and 27.

Claim 10 correspond to previous claims 14, 16, 32, 34, 36, 37 and 38.

Claim 11 corresponds to previous claim 35.

Claim 12 corresponds to previous claim 33.

Claim 13 corresponds to previous claim 20.

Previous claims 11, 17-19, 25, 26, 28-31, 39 and 40 have been deleted.

The amended set of claims should thus be allowable under Art. 123(2) EPC.

Dr. Jörg Helbing
(European Patent Attorney)

Enclosure (in triplicate)
Set of claims (1-13)

CLAIMS:

1. A DNA comprising a nucleic acid sequence selected from the group consisting of:

(a) nucleic acid sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4;

(b) nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 encoding at least a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4;

(c) nucleic acid sequence comprising at least 20 consecutive bases of a nucleic acid sequence of (a) or (b) and encoding a polypeptide having APRIL activity;

(d) nucleic acid sequences the complementary strand of which hybridizes to a nucleic acid sequence of any one of (a) to (c) and being at least 70% homologous with an active site of a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4;

(e) nucleic acid sequences encoding a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 with one or more conservative substitutions, alteration or deletions;

(f) nucleic acid sequences encoding a soluble fragment of a polypeptide comprising amino acids 110 to 250 as shown in Figure 1A; and

(g) nucleic acid sequences encoding a fragment of a polypeptide comprising amino acid residues 117 to 250 as shown in Figure 1B or the amino acid residues 29 to 49 (transmembrane) as shown in Figure 1A.

2. A recombinant DNA molecule comprising the DNA of claim 1 which is operatively linked to an expression control sequence.

3. A unicellular host transformed with a recombinant DNA molecule of claim 2.

4. A method of producing substantially pure APRIL comprising the step of culturing the unicellular host of claim 3.

5. A polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4;

(b) a soluble biologically active fragment of claim (a);

(c) the amino acid sequence of (a) with one or more conservative amino acid substitution; and

(d) amino acid residues 110 to 250 as shown in Figure 1A, amino acid residues 117 to 250 as shown in Figure 1B or the amino acid residues 29 to 49 (transmembrane) as shown in Figure 1A,
or obtainable by the method of claim 4.

6. A polypeptide, the amino acid sequence of which consistis of SEQ ID NO:2 or SEQ ID NO:4.

7. An antibody preparation comprising antibodies that are reactive to the polypeptide of claim 5 or 6.

8. The antibody preparation of claim 7 where in the antibody is a monoclonal antibody.

9. A pharmaceutical composition comprising the antibody preparation of claim 7 or 8 or a therapeutically effective amount of the polypeptide of claim 5 or 6 and a pharmaceutically acceptable carrier.

10. Use of an anti-APRIL receptor antibody or the antibody preparation of claim 7 or 8 r the polypeptide of claim 5 or 6 for the preparation of a medicament for preventing or reducing the severity of an autoimmune

disease or an immune response to a tissue graft in a mammal or treating, suppressing or altering the progression of cancer or for inducing cell death in a mammal.

11. The method of claim 10 wherein said immune response involves human carcinoma cells.

12. The use of claim 10 further comprising the administration of interferon-γ.

13. A method for identifying a receptor for APRIL comprising:

(a) providing APRIL or a fragment thereof;

(b) labeling said APRIL or fragment thereof with a detectable label; and

(c) screening a composition to detect receptors which bind to the detectably labeled of step b.

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Section de Dépôt

COPY

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Datum/Date
17.05.00

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
JH/m1000789ep	98946066.2-2105/

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Apotech R&D S.A.

C O M M U N I C A T I O N

concerning the registration of amendments relating to

[X] a transfer (Rule 20/Rules 61,20 EPC)

[ ] entries pertaining to the applicant/the proprietor (Rule 92(1)(f) EPC)

As requested, the entries pertaining to the applicant of the above-mentioned European patent application/to the proprietor of the above-mentioned European patent have been amended to the following:

AT-BE-CH-CY-DE-DK-ES-FI-FR-GB-GR-IE-IT-LI-LU-MC-NL-PT-SE
Apotech R&D S.A.
84, rue du Rhône
1204 Genève/CH

The registration of the changes has taken effect on 09.05.00

In the case of a published application/a patent, the change will be recorded in the Register of European Patents and published in the European Patent Bulletin (Section I.12/II.12).

Your attention is drawn to the fact that, in the case of the registration of a transfer, any automatic debit order only ceases to be effective from the date of its express revocation (cf. point 14(c) of the Arrangements for the automatic debiting procedure, Supplement to OJ EPO 6/1994).

Formalities officer
Tel.: (+49-89) 2399-2384

H. Caspelherr

EUROPEAN PATENT OFFICE - OFFICE EUROPÉEN DES BREVETS - EUROPÄISCHES PATENTAMT - DEN HAAG - THE HAGUE - LA HAYE

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58

09. Mai 2000

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Dipl.-Chem. Dr. Thomas Weber
Dipl.-Chem. Dr. Jörg Helbing
Dipl.-Ing. Alexander von Kirschbaum

Our Ref.:
000773ep/JH/ml
000789ep/JH/ml

Cologne,
May 08, 2000

European patent applications 98946966.3, 98946066.2 and 98946052.2
Assignment from Biogen, Inc. to Apotech R&D S.A.

CH 11. 05. 00

The European patent applications 98946966.3 and 98946066.2 shall be assigned from Biogen, Inc to

APPR 801 8010.3

Apotech R&D S.A.
84 Rue du Rhone
1204 Geneva
CH.

Please find enclosed the executed assignment. The fee for the registration of the transfer of EUR 152.00 shall be debited to our account No. 2800 0007.

Concerning application No. 98946052.2 (for which the assignment, which was requested in the PCT proceedings, was already recorded), it is to be noted that the correct designation of the assignee is "Apotech R&D S.A." as can be seen from the enclosed assignment. This should be recorded.

Patent Attorney

(Dr. Jörg Helbing)
Enclosure

ASSIGNMENT

We

Biogen Inc.
14 Cambridge Center
Cambridge, MA 02142
USA

~~herewith declare to have assigned all titles and rights from our European Patent~~

Applications

98946066.2 (title: "April - a Novel Protein with Growth Effects")

98946052.2 (title: "Kay - a Novel Immune System Protein")

98946966.3 (title: "Cysteine Rich Receptors: Trail"

to

Apotech R&D S.A.
84 Rue du Rhone
1204 Geneva
CH

and request to have the assignment registered accordingly.

This 1st day of May 2000

.....

(Name and position of signatory)
Raymond Arner
Assistant Secretary
Biogen, Inc.

We accept the above assignment.

This 18 day of April 2000

.....

(Name and position of signatory)
Mr. Henry Verrey
Chief Executive Officer
Apotech R&D S.A.

An das Europäische Patentamt | To the European Patent Office | A l'Office européen des brevets

EPO - Munich 22

05. April 2000

Eintritt in die regionale Phase vor dem EPA als Bestimmungsamt oder ausgewähltem Amt

Entry into the regional phase before the EPO as designated or elected Office

Entrée dans la phase régionale devant l'OEB agissant en qualité d'office désigné ou élu

Europäische Anmeldenummer oder, falls nicht bekannt, PCT-Aktenzeichen oder PCT-Veröffentlichungsnummer	European application number, or, if not known, PCT application or publication number	Numéro de dépôt de la demande de brevet européen ou, à défaut, numéro de dépôt PCT ou de publication PCT
	98946066.2 (PCT/US98/19191)	
Zeichen des Anmelders oder Vertreters (max. 15 Positionen)	Applicant's or representative's reference (max. 15 spaces)	Référence du demandeur ou du mandataire (15 caractères ou espaces au maximum)
	JH/ml 000789ep	

☒ ☐ **1. Anmelder**
Die Angaben über den (die) Anmelder sind in der internationalen Veröffentlichung enthalten oder vom Internationalen Büro nach der internationalen Veröffentlichung vermerkt werden.

Änderungen, die das Internationale Büro noch nicht vermerkt hat, sind auf einem Zusatzblatt angegeben.

Zustellanschrift
(siehe Merkblatt II, 1)

1. Applicant
Indications concerning the applicant(s) are contained in the international publication or recorded by the International Bureau after the international publication.

Changes which have not yet been recorded by the International Bureau are set out on an additional sheet.

Address for correspondence
(see Notes II, 1)

1. Demandeur
Les indications concernant le(s) demandeur(s) figurent dans la publication internationale ou ont été enregistrées par le Bureau international après la publication internationale.

Les changements qui n'ont pas encore été enregistrés par le Bureau international sont indiqués sur une feuille additionnelle.

Adresse pour la correspondance
(voir notice II, 1)

2. Vertreter

Name (Nur einen Vertreter angeben, der in das europäische Patentregister eingetragen und an den zugestellt wird)

Geschäftsanschrift

Telefon

Telefax **Telex**

2. Representative

Name (Name **only one** representative who will be listed in the Register of European Patents and to whom notification will be made)

Dr. Jörg Helbing

Address of place of business

Von Kreisler Selting Werner
P.O. Box 10 22 41
D-50462 Köln

Telephone

0221/91 65 20

Fax **Telex**

0221/13 42 97

2. Mandataire

Nom (N'indiquer **qu'un seul** mandataire, qui sera inscrit au Registre européen des brevets et auquel signification sera faite)

Adresse professionnelle

Téléphone

Téléfax **Télex**

☒ Weitere(r) Vertreter auf Zusatzblatt

Additional representative(s) on additional sheet

Autre(s) mandataire(s) sur une feuille additionnelle

3. Vollmacht

☐ Einzelvollmacht ist beigefügt.

☐ Allgemeine Vollmacht ist registriert unter Nummer:

☐ Allgemeine Vollmacht ist eingereicht, aber noch nicht registriert.

☐ Die beim EPA als PCT-Anmeldeamt eingereichte Vollmacht schließt ausdrücklich die regionale Phase ein.

3. Authorisation

Individual authorisation is attached.

General authorisation has been registered under No:

(A) € 4022,50 Zur Kasse

A general authorisation has been filed, but not yet registered.

The authorisation filed with the EPO as PCT receiving Office expressly includes the regional phase.

3. Pouvoir

Un pouvoir spécial est joint.

Un pouvoir général a été enregistré sous le n° :

Un pouvoir général a été déposé, mais n'est pas encore enregistré.

Le pouvoir général déposé à l'OEB agissant en qualité d'office récepteur au titre du PCT s'applique expressément à la phase régionale.

☒ **4. Prüfungsantrag**
Hiermit wird die Prüfung der Anmeldung gemäß Art. 94 EPÜ beantragt. Die Prüfungsgebühr wird (wurde) entrichtet.

Prüfungsantrag in einer zugelassenen Nichtamtssprache (siehe Merkblatt III, 6.2):

4. Request for examination
Examination of the application under Art. 94 EPC is hereby requested. The examination fee is being (has been, will be) paid.

Request for examination in an admissible non-EPO language (see Notes III, 6.2):

4. Requête en examen
Il est demandé que soit examinée la demande de brevet conformément à l'art. 94 CBE. Il est (a été, sera) procédé au paiement de la taxe d'examen.

Requête en examen dans une langue non officielle autorisée (voir notice III, 6.2):

☐ **5. Abschriften**
Zusätzliche Abschrift(en) der im erganzenden europäischen Recherchenbericht angeführten Schriftstücke wird (werden) beantragt.

Anzahl der **zusätzlichen** Sätze von Abschriften

5. Copies
Additional copy (copies) of the documents cited in the supplementary European search report is (are) requested.

Number of **additional** sets of copies

5. Copies
Prière de fournir une ou plusieurs copies supplémentaires des documents cités dans le rapport complémentaire de recherche européenne.

Nombre de jeux **supplémentaires** de copies

6. Für das Verfahren vor dem EPA bestimmte Unterlagen

6.1 Dem Verfahren vor dem EPA als **Bestimmungsamt** (PCT I) sind folgende Unterlagen zugrunde zu legen:

☒ die vom Internationalen Büro **veröffentlichten Anmeldungsunterlagen** (mit **allen** Ansprüchen, Beschreibung und Zeichnungen), gegebenenfalls mit den geänderten Ansprüchen nach Art. 19 PCT

☐ soweit sie nicht ersetzt werden durch die **in drei Stücken** beigefügten **Änderungen.**

Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!

6. Documents intended for proceedings before the EPO

6.1 Proceedings before the EPO as **designated Office** (PCT I) are to be based on the following documents:

the **application documents published** by the International Bureau (with **all** claims, description and drawings), where applicable with amended claims under Art. 19 PCT

unless replaced by the **amendments** enclosed **in triplicate.**

Where necessary, clarifications must be submitted on a separate sheet!

6. Pièces destinées à la procédure devant l'OEB

6.1 La procédure devant l'OEB agissant en qualité **d'office désigné** (PCT I) doit se fonder sur les pièces suivantes :

les **pièces de la demande publiée** par le Bureau international (avec **toutes** les revendications, la description et les dessins), éventuellement avec les revendications modifiées conformément à l'article 19 du PCT

dans la mesure où elles ne sont pas remplacées par les **modifications** jointes **en trois exemplaires.**

Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!

6.2 Dem Verfahren vor dem EPA als **ausgewähltem Amt** (PCT II) sind folgende Unterlagen zugrunde zu legen:

☒ die dem **internationalen vorläufigen Prüfungsbericht zugrunde gelegten Unterlagen,** einschließlich seiner eventuellen **Anlagen**
(Solche Anlagen müssen immer in drei Stücken beigefügt werden)

☒ soweit sie nicht ersetzt werden durch die **in drei Stücken** beigefügten **Änderungen.**

Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!

☒ Sind dem EPA als mit der internationalen vorläufigen Prüfung beauftragten Behörde **Versuchsberichte** zugegangen, dürfen diese dem Verfahren vor dem EPA zugrunde gelegt werden.

6.2 Proceedings before the EPO as **elected Office** (PCT II) are to be based on the following documents:

the **documents on which the international preliminary examination report is based,** including its possible **annexes**
(Such annexes must always be filed in triplicate)

unless replaced by the **amendments** enclosed **in triplicate.**
see additional sheet

Where necessary, clarifications must be submitted on a separate sheet!

If the EPO as International Preliminary Examining Authority has received **test reports,** these may be used as the basis of proceedings before the EPO.

6.2 La procédure devant l'OEB **agissant en qualité d'office élu** (PCT II) doit se fonder sur les pièces suivantes :

les **pièces sur lesquelles se fonde le rapport d'examen préliminaire international,** y compris ses **annexes** éventuelles
(De telles annexes sont toujours à joindre en trois exemplaires)

dans la mesure où elles ne sont pas remplacées par les **modifications** jointes **en trois exemplaires.**

Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!

Si l'OEB, agissant en qualité d'administration chargée de l'examen préliminaire international, a reçu des **rapports d'essais,** ceux-ci peuvent constituer la base de la procédure devant l'OEB.

7. Übersetzungen
Beigefügt sind die nachfolgend angekreuzten Übersetzungen in einer der Amtssprachen des EPA (Deutsch, Englisch, Französisch):

7. Translations
Translations in one of the official languages of the EPO (English, French, German) are enclosed as crossed below:

7. Traductions
Vous trouverez ci-joint, les traductions cochées ci-après dans l'une des langues officielles de l'OEB (allemand, anglais, français) :

- *Im Verfahren vor dem EPA als **Bestimmungsamt oder ausgewähltem Amt** (PCT I + II):*

☐ Übersetzung der **ursprünglich eingereichten internationalen Anmeldung** (Beschreibung, Ansprüche, etwaige Textbestandteile in den Zeichnungen), der veröffentlichten Zusammenfassung, und etwaiger Angaben über Mikroorganismen nach Regel 13bis.3 und 13bis.4 PCT, **in drei Stücken**

☐ Übersetzung der **prioritätsbegründenden Anmeldung(en), in einem Stück**

- *In proceedings before the EPO as **designated or elected Office** (PCT I + II):*

Translation of the **international application** (description, claims, any text in the drawings) **as originally filed**, of the abstract as published and of any indication under Rule 13bis.3 and 13bis.4 PCT regarding micro-organisms, **in triplicate**

Translation of **the priority application(s), in one copy**

- *Dans la procédure devant l'OEB agissant en qualité d'**office désigné ou élu** (PCT I + II):*

Traduction de la **demande internationale telle que déposée initialement** (description, revendications, textes figurant éventuellement dans les dessins), de l'abrégé publié, et de toutes indications visées aux règles 13bis.3 et 13bis.4 du PCT concernant les micro-organismes, **en trois exemplaires**

Traduction de la (des) **demande(s) ouvrant le droit de priorité, en un exemplaire**

- ***Zusätzlich** im Verfahren vor dem EPA als **Bestimmungsamt** (PCT I):*

☐ Übersetzung der nach Art. 19 PCT **geänderten Ansprüche** nebst Erklärung, falls diese dem Verfahren vor dem EPA zugrunde gelegt werden sollen (siehe Feld 6), **in drei Stücken**

- ***In addition**, in proceedings before the EPO as **designated Office** (PCT I):*

Translation of **amended claims** and any statement under Art. 19 PCT, if the claims as amended are to form the basis for the proceedings before the EPO (see Section 6), **in triplicate**

- ***De plus**, dans la procédure devant l'OEB agissant en qualité d'**office désigné** (PCT I) :*

Traduction des **revendications modifiées** et de la déclaration faite conformément à l'article 19 du PCT, si la procédure devant l'OEB doit être fondée sur les revendications modifiées (voir la rubrique 6), **en trois exemplaires**

- ***Zusätzlich** im Verfahren vor dem EPA als **ausgewähltem Amt** (PCT II):*

☐ Übersetzung der **Anlagen zum internationalen vorläufigen Prüfungsbericht, in drei Stücken**

- ***In addition**, in proceedings before the EPO as **elected Office** (PCT II):*

Translation of any **annexes to the international preliminary examination report, in triplicate**

- ***De plus**, dans la procédure devant l'OEB agissant en qualité d'**office élu** (PCT II) :*

Traduction des **annexes du rapport d'examen préliminaire international, en trois exemplaires**

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8. Biologisches Material
☐ Die Erfindung bezieht sich auf bzw. verwendet biologisches Material, das nach Regel 28 EPÜ hinterlegt worden ist.

☐ Die **Angaben nach Regel 28(1)c) EPÜ** (falls noch nicht bekannt, die Hinterlegungsstelle und das (die) Bezugszeichen [Nummer, Symbole usw.] des Hinterlegers) sind in der internationalen Veröffentlichung oder in der gemäß Feld 7 eingereichten Übersetzung enthalten auf:

Seite(n) / Zeile(n)

8. Biological material
The invention relates to and/or uses biological material deposited under Rule 28 EPC.

The **particulars referred to in Rule 28(1)(c) EPC** (if not yet known, the depository institution and the identification reference(s) [number, symbols etc.] of the depositor) are given in the international publication or in the translation submitted under Section 7 on:

page(s) / line(s)

8. Matière biologique
L'invention concerne et/ou utilise de la matière biologique, déposée conformément à la règle 28 CBE.

Les **indications visées à la règle 28(1)c) CBE** (si pas encore connues, l'autorité de dépôt et la (les) référence(s) d'identification [numéro ou symboles etc.] du déposant) figurent dans la publication internationale ou dans une traduction produite conformément à la rubrique 7 à la / aux:

page(s) / ligne(s)

Die **Empfangsbescheinigung(en)** der Hinterlegungsstelle

☐ ist (sind) beigefügt

☐ wird (werden) nachgereicht

☐ Verzicht auf die Verpflichtung des Antragstellers nach Regel 28(3) auf gesondertem Schriftstück

The **receipt(s) of deposit** issued by the depositary institution

is (are) enclosed

will be filed at a later date

Waiver of the right to an undertaking from the requester pursuant to Rule 28(3) attached.

Le(s) **récépissé(s) de dépôt** délivré(s) par l'autorité de dépôt

est (sont) joint(s)

sera (seront) produit(s) ultérieurement

Renonciation, sur document distinct, à l'engagement du requérant au titre de la règle 28(3).

9. Nucleotid- und Aminosäuresequenzen

☒ Die nach Regeln 5.2 und 13ter PCT sowie Regel 104b (3a) EPÜ erforderlichen Unterlagen liegen dem EPA bereits vor.

☐ Das schriftliche Sequenzprotokoll wird anliegend in einer Amtssprache des EPA nachgereicht.

☐ Das Sequenzprotokoll geht nicht über den Inhalt der Anmeldung in der ursprünglich eingereichten Fassung hinaus.

☐ Der vorgeschriebene maschinenlesbare Datenträger ist beigefügt.

☐ Die auf dem Datenträger gespeicherte Information stimmt mit dem schriftlichen Sequenzprotokoll überein.

9. Nucleotide and amino acid sequences

The items necessary in accordance with Rules 5.2 and 13ter PCT and Rule 104b (3a) EPC have already been furnished to the EPO.

The written sequence listing is furnished herewith in an official language of the EPO.

The sequence listing does not include matter which goes beyond the content of the application as filed.

The prescribed machine-readable data carrier is enclosed.

The information recorded on the data carrier is identical to the written sequence listing.

9. Séquences de nucléotides et d'acides aminés

Les pièces requises selon les règles 5.2 et 13ter PCT et la règle 104ter (3bis) CBE ont déjà été déposées auprès de l'OEB.

La liste de séquences écrite est produite ci-joint dans une des langues officielles de l'OEB.

La liste de séquences ne contient pas d'éléments s'étendant au-delà du contenu de la demande telle qu'elle a été déposée.

Le support de données prescrit, déchiffrable par machine, est annexé.

L'information figurant sur le support de données est identique à celle que contient la liste de séquences écrite.

10. Benennungsgebühren

10.1 Benennungsgebühren werden für nachstehende **in der internationalen Anmeldung bestimmte Vertragsstaaten** des EPÜ entrichtet:

10. Designation fees

10.1 Designation fees are paid in respect of the following EPC **Contracting States designated in the international application** for a European patent:

10. Taxes de désignation

10.1 Les taxes de désignation sont acquittées pour ceux des **Etats contractants** de la CBE **désignés dans la demande internationale** qui sont indiqués ci-après:

		Österreich	Austria	Autriche
☒	AT			
☒	BE	Belgien	Belgium	Belgique
☒	CH/LI	Schweiz und Liechtenstein	Switzerland and Liechtenstein	Suisse et Liechtenstein
☒	CY	Zypern [1]	Cyprus [1]	Chypre [1]
☒	DE	Deutschland	Germany	Allemagne
☒	DK	Dänemark	Denmark	Danemark
☒	ES	Spanien	Spain	Espagne
☒	FI	Finnland	Finland	Finlande
☒	FR	Frankreich	France	France
☒	GB	Vereinigtes Königreich	United Kingdom	Royaume-Uni
☒	GR	Griechenland	Greece	Grèce
☒	IE	Irland	Ireland	Irlande
☒	IT	Italien	Italy	Italie
☒	LU	Luxemburg	Luxembourg	Luxembourg
☒	MC	Monaco	Monaco	Monaco
☒	NL	Niederlande	Netherlands	Pays-Bas
☒	PT	Portugal	Portugal	Portugal
☒	SE	Schweden	Sweden	Suède
☐	____	_____ [2]	_____ [2]	_____ [2]
☐	____	_____ [2]	_____ [2]	_____ [2]

☒ 10.2 Derzeit ist nicht beabsichtigt, Benennungsgebühren für die in Feld 10.1 nicht angekreuzten, aber in der internationalen Anmeldung bestimmten Vertragsstaaten des EPÜ zu entrichten. Insoweit wird auf die Zustellung einer Mitteilung nach Regel 85a(1) EPÜ verzichtet. Sofern diese Benennungsgebühren nicht bis zum Ablauf der in Regel 85a(2) EPÜ vorgesehenen Nachfrist entrichtet werden, wird beantragt, von einer Mitteilung nach Regel 69(1) EPÜ abzusehen.

10.2 At present it is not intended to pay designation fees for the EPC Contracting States not marked with a cross under 10.1 but designated in the international application. No communication under Rule 85a(1) EPC in respect of these designation fees need be notified. If they have not been paid by the time the period of grace allowed in Rule 85a(2) EPC expires, it is requested that no communication be sent under Rule 69(1) EPC.

10.2 Il n'est pas actuellement envisagé d'acquitter les taxes de désignation pour les Etats contractants de la CBE qui ne sont pas cochés sous la rubrique 10.1, mais qui sont désignés dans la demande internationale. Le demandeur renonce ainsi à la notification prévue à la règle 85bis(1) CBE. Si ces taxes de désignation ne sont pas acquittées à l'expiration du délai supplémentaire prévu à la règle 85bis(2) CBE, il est demandé de s'abstenir d'envoyer une notification, établie conformément à la règle 69(1) CBE.

1) Nur möglich, falls in der internationalen Anmeldung am oder nach dem 1. April 1998 bestimmt.
2) Vorgesehen für die Eintragung weiterer Vertragsstaaten des EPÜ, für die der PCT oder das EPÜ nach Drucklegung dieses Formblatts in Kraft tritt, und die in der internationalen Anmeldung für ein europäisches Patent bestimmt waren.

1) Only possible if designated in the international application on or after 1 April 1998
2) Space for any other EPC Contracting States which may become PCT or EPC Contracting States after this form has been printed and which were designated for a European patent in the international application.

1) Seulement possible, si désignée dans la demande internationale au 1er avril 1998 ou après cette date
2) Prévu pour l'inscription d'autres Etats contractants de la CBE à l'égard desquels le PCT ou la CBE entrera en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale pour un brevet européen

☒ **11. Erstreckung des europäischen Patents**

Diese Anmeldung gilt auch als Erstreckungsantrag hinsichtlich aller **in der internationalen Anmeldung bestimmten Nicht-Vertragsstaaten** des EPÜ, mit denen bei Einreichung der internationalen Anmeldung »Erstreckungsabkommen« in Kraft waren*.
Die Erstreckung wird jedoch nur wirksam, wenn die vorgeschriebene Erstreckungsgebühr entrichtet wird.
Der Anmelder beabsichtigt, die Erstreckungsgebühr für die nachfolgend angekreuzten Staaten zu entrichten:

☒	SI	Slowenien (* ab 1. März 1994)	Slovenia (* as of 1 March 1994)
☒	LT	Litauen (* ab 5. Juli 1994)	Lithuania (* as of 5 July 1994)
☒	LV	Lettland (* ab 1. Mai 1995)	Latvia (* as of 1 May 1995)
☒	AL	Albanien (* ab 1. Februar 1996)	Albania (* as of 1 February 1996)
☒	RO	Rumänien (* ab 15. Oktober 1996)	Romania (* as of 15 October 1996)
☒	MK	Ehemalige jugoslawische Republik Mazedonien (* ab 1 November 1997)	Former Yugoslav Republic of Macedonia (* as of 1 November 1997)
☐	____	_____ [1]	_____ [1]

1) Platz für Staaten, mit denen »Erstreckungsabkommen« nach Drucklegung dieses Formblatts in Kraft treten und die in der internationalen Anmeldung bestimmt waren

11. Extension of the European patent

This application is also considered as being a request for extension to all the **non-Contracting States** to the EPC **designated in the international application** with which "extension agreements" were in force on the date of filing the international application*.
However, the extension only takes effect if the prescribed extension fee is paid.
The applicant intends to pay the extension fee for the States marked with a cross below:

Slovenia (* as of 1 March 1994)
Lithuania (* as of 5 July 1994)
Latvia (* as of 1 May 1995)
Albania (* as of 1 February 1996)
Romania (* as of 15 October 1996)
Former Yugoslav Republic of Macedonia (* as of 1 November 1997)
_____ [1]

1) Space for States with which "extension agreements" enter into force after this form has been printed and which were designated in the international application.

11. Extension des effets du brevet européen

La présente demande est également réputée demande d'extension à tous les **Etats non contractants de** la CBE **désignés dans la demande internationale**, avec lesquels existaient, lors du dépôt de la demande, des «accords d'extension»* **Toutefois, l'extension ne produit ses effets que si la taxe d'extension prescrite est acquittée.**
Le demandeur se propose actuellement d'acquitter la taxe d'extension pour les Etats dont le nom est coché ci-après:

Slovénie (* à compter du 1er mars 1994)
Lituanie (* à compter du 5 juillet 1994)
Lettonie (* à compter du 1er mai 1995)
Albanie (* à compter du 1er février 1996)
Roumanie (* à compter du 15 octobre 1996)
Ex-République yougoslave de Macédoine (* à compter du 1er novembre 1997)
_____ [1]

1) Prévu pour des Etats à l'égard desquels des «accords d'extension» entreront en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale

☐ **12. Automatischer Abbuchungsauftrag** ***(Nur möglich für Inhaber von beim EPA geführten laufenden Konten)***

Das EPA wird beauftragt, nach Maßgabe der Vorschriften über das automatische Abbuchungsverfahren fällige Gebühren und Auslagen vom untenstehenden laufenden Konto abzubuchen.

Nummer des laufenden Kontos / Name des Kontoinhabers

_____

12. Automatic debit order ***(for EPO deposit account holders only)***

The EPO is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account below any fees and costs falling due.

Deposit account number / Account holder's name

_____

12. Ordre de prélèvement automatique ***(uniquement possible pour les titulaires de comptes courants ouverts auprès de l'OEB)***

Par la présente, il est demandé à l'OEB de prélever du compte courant ci-dessous les taxes et frais venant à échéance, conformément à la réglementation relative au prélèvement automatique.

N° du compte courant / Nom du titulaire du compte

_____

☒ **13.** Eventuelle **Rückzahlungen** auf das beim EPA geführte laufende Konto Nummer

_____

Name des Kontoinhabers

_____

13. Reimbursement, if any, to EPO deposit account number

2800 0007

Account holder's name

Dr. Jörg Helbing

13. Remboursements éventuels à effectuer sur le compte courant ouvert auprès de l'OEB numéro

_____

Nom du titulaire du compte

_____

14. Unterschrift(en) des (der) Anmelder(s) oder Vertreters

Ort / Datum

14. Signature(s) of applicant(s) or representative

Place / Date

Cologne, April 03, 2000

(Dr. Jörg Helbing)

14. Signature(s) du (des) demandeur(s) ou du mandataire

Lieu / Date

Für Angestellte (Art. 133(3) EPÜ) mit allgemeiner Vollmacht:

Nr. _____

Name(n) des (der) Unterzeichneten bitte mit Schreibmaschine wiederholen. Bei juristischen Personen bitte auch die Stellung des (der) Unterzeichneten innerhalb der Gesellschaft eintragen

For employees (Art. 133(3) EPC) having a general authorisation:

No. _____

Please type name(s) under signature(s). In the case of legal persons, the position of the signatory within the company should also be typed.

Pour les employés (art. 133(3) CBE) disposant d'un pouvoir général :

N° _____

Veuillez faire figurer le nom dactylographié sous la signature. Si ce nom désigne une personne morale, ajouter la mention dactylographiée de la position occupée par le signataire au sein de la société

Additional sheet

Representatives (continued):

Dipl.-Chem. Alek von Kreisler
Dipl.-Ing. Günther Selting
Dipl.-Chem. Dr. Hans-Karsten Werner
Dipl.-Chem. Dr. Johann F. Fues
Dipl.-Ing. Georg Dallmeyer
Dipl.-Ing. Jochen Hilleringmann
Dipl.-Chem. Dr. Hans-Peter Jönsson
Dipl.-Chem. Dr. Hans-Wilhelm Meyers
Dipl.-Chem. Dr. Thomas Weber
Dipl.-Ing. Alexander von Kirschbaum

Box 6.2:

Enclosed is a set of claims (1-40) on which the present case shall be further prosecuted. The new set of claims is identical with the set of claims as originally filed in the PCT procedure.

I claim:

1. A DNA sequence encoding APRIL or a fragment thereof.

2. A DNA sequence encoding APRIL said sequence consisting essentially of SEQ. ID. NO.2

3. A DNA sequence consisting essentially of SEQ. ID. NO. 1 said DNA encoding a polypeptide, said polypeptide consisting essentially of SEQ. ID. NO. 2.

4. A DNA sequence that hybridizes to at least a fragment of SEQ. ID NO. 1 said fragment comprising at least 20 consecutive bases, said DNA sequence encoding a polypeptide that is at least 30% homologous with an active site of APRIL.

5. A DNA sequence according to claim 2 wherein said sequence consists essentially of SEQ. ID. NO. 1 with conservative substitutions, alterations or deletions.

6. A recombinant DNA molecule comprising a DNA sequence encoding APRIL said sequence operatively linked to an expression control sequence.

7. The molecule of claim 6 comprising SEQ. ID. NO. 1.

8. A unicellular host transformed with a recombinant DNA molecule of claim 6 or 7.

9. A DNA sequence encoding APRIL having the amino acid sequence of SEQ. ID. NO. 2.

10. A method for producing substantially pure APRIL comprising the step of culturing the unicellular host of claim 8.

11. APRIL essentially free of normally associated animal proteins.

12. The APRIL of claim 11 consisting essentially of SEQ. ID. NO. 2.

13. A pharmaceutical composition comprising a therapeutically effective amount of APRIL or an active fragment thereof, and a pharmaceutically acceptable carrier.

14. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

15. The pharmaceutical composition of claim 13 wherein said APRIL or active fragment thereof comprises SEQ. ID. NO. 2 or a biologically active fragment thereof.

16. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

17. A method for stimulating the immune system comprising administering the composition of claim 13.

18. A method for suppressing the immune system comprising administering an effective amount of the pharmaceutical composition according to claim 13.

19. A method for treating cancer comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 13.

20. A method for identifying a receptor for APRIL comprising:

a. providing APRIL or a fragment thereof,

b. labeling said APRIL or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled of step b.

21. A soluble biologically active fragment of the APRIL of claim 11.

22. A polypeptide comprising an amino acid sequence that is encoded by a DNA selected from the group consisting of:

a. a DNA sequence comprising SEQ. ID. NO. 1;

b. a DNA sequence that hybridizes to the DNA defined in a. and coding on expression for a polypeptide that is at least 40% homologous with the APRIL of claim 12.

23. An antibody preparation that is reactive to APRIL or its receptor or biologically active fragments thereof.

24. The antibody preparation of claim 23 comprising monoclonal antibodies.

25. A method for producing an antibody preparation reactive to APRIL or its receptor comprising the step of immunizing an organism with APRIL or its receptor, or an antigenic fragment thereof.

26. An antisense nucleic acid against APRIL comprising a nucleic acid sequence hybridizing to at least a portion of SEQ. ID. NO. 1.

27. A pharmaceutical composition comprising an antibody preparation according to claim 24.

28. A method of expressing APRIL in a mammalian cell comprising:

a. introducing a gene encoding APRIL into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

29. A method of treating a disorder related to APRIL in a mammal

a. introducing into a cell a therapeutically effective amount of a vector comprising a gene encoding APRIL; and

b. expressing said gene in said mammalian cell.

30. The method of claim 29 wherein the mammal is a human.

31. The method of claim 29 wherein said vector is a virus.

32. A method of inducing cell death comprising the administration of an agent capable of interfering with the binding of APRIL to a receptor.

33. The method of claim 32 further comprising the administration of interferon-γ.

34. A method of treating, suppressing, activating or altering an immune response involving a signaling pathway between APRIL and its receptor, said method comprising the step of administering an effective amount of a blocking agent capable of interfering with the association between APRIL and its receptor.

35. The method of claim 34 wherein said immune response involves human carcinoma cells.

36. A method of treating, suppressing or altering the progression of a cancer comprising adminstering to a patient an effective amount of a blocking agent between April and its receptor capable of interfering with the association.

37. The method of claim 36, wherein the blocking agent is a modified inhibitory form of APRIL, or anti-APRIL antibodies or biologically active fragments thereof.

38. The method of Claim 37 wherein the blocking agent is an anti-APRIL receptor antibody.

39. The method of Claim 36 wherein the blocking agent is administered to a patient in combination with at least one chemotherapeutic agent.

40. The method of claim 39 further comprising the step of administering radiation therapy to said patient.

What is claimed is:

1. Nucleic acid encoding an APRIL ligand, said ligand comprising a polypeptide of at least about 102 amino acids.

2. Nucleic acid of claim 1 encoding SEQ ID No:2.

3. Nucleic acid encoding an APRIL ligand or a fragment thereof, wherein said ligand comprises an amino acid sequence found in amino acids 1 to 55 of SEQ ID NO:2.

4. Nucleic acid encoding an APRIL ligand or a fragment thereof, wherein said ligand comprises an amino acid sequence found in amino acids 157 to 250 of SEQ ID NO:2.

5. Nucleic acid of claims 1-4 encoding an amino acid substitution analog of SEQ ID No:2.

6. Nucleic acid of claim 5 wherein said substitution analog, when aligned with SEQ ID No:2, shares at least 40% sequence similarity therewith, further wherein said substitution analog shares at least 80% of aligned cysteine residues with said APRIL ligand.

7. Nucleic acid of claim 5 wherein said substitution analog, when aligned with SEQ ID No:2, shares at least 80% sequence similarity therewith.

8. Nucleic acid having a nucleotide sequence comprising

(a) SEQ ID NO:1; or

(b) a substitution analog of SEQ ID NO:1; or

(c) an alteration analog of SEQ ID NO:1; or

(d) a deletion analog of SEQ ID NO:1.

9. A vector having the nucleic acid of claim 1, 2, 3, 4, or 8, present as an insert therein, said vector optionally comprising an expression control sequence operably linked to said insert.

10. A host cell comprising the vector of claim 9.

11. A method for producing substantially pure APRIL comprising the steps of: culturing the host cells of claim 10.

12. An APRIL ligand polypeptide comprising at least about 102 amino acids.

13. An APRIL ligand polypeptide comprising an amino acid sequence found in amino acids 1 to 55 of SEQ ID NO:2 or a fragment thereof.

14. An APRIL ligand polypeptide comprising an amino acid sequence found in amino acids 157 to 250 of SEQ ID NO:2 or a fragment thereof.

15. An APRIL ligand selected from:

(a) SEQ ID No:2; or

(b) an amino acid substitution analog of SEQ ID No:2.

16. A soluble APRIL ligand polypeptide of claims 12-15.

17. A pharmaceutical composition comprising a therapeutically effective amount of an APRIL ligand polypeptide of claims 12-15 and a pharmaceutically acceptable carrier.

19. An antibody that binds specifically to an APRIL ligand polypeptide of claim 12, 13, 14, 15 or 16.

20. An antibody that blocks binding of an APRIL ligand polypeptide to an APRIL receptor polypeptide.

21. An antibody of claim 20 that binds specifically to an APRIL polypeptide.

22. The use of a pharmaceutical composition of claim 17 for the preparation of a medicament for preventing or reducing the severity of an autoimmune disease; preventing or reducing the severity of an immune response to a tissue graft; stimulating or suppressing the immune system; or treating cancer.

23. A method for identifying a receptor for APRIL comprising:

a. providing APRIL or a fragment thereof,

b. labeling said APRIL or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled of step b.

24. A method of expressing APRIL in a mammalian cell comprising:

a. introducing a gene encoding APRIL into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

25. The use of a vector comprising a gene encoding APRIL for the preparation of a medicament for treating a disorder related to APRIL in a mammal comprising

a. introducing into a cell a therapeutically effective amount of said vector; and

b. expressing said gene in said mammalian cell.

26. The method of claim 25 wherein the mammal is a human.

27. The method of claim 25 wherein said vector is a virus.

28. The use of an agent capable of interfering with the binding of APRIL to a receptor for the preparation of a medicament for inducing cell death.

29. The method of claim 28 further comprising the administration of interferon-γ.

30. The use of a blocking agent capable of interfering with the association between APRIL and its receptor for the preparation of a medicament for treating, suppressing, activating or altering an immune response involving a signaling pathway between APRIL and its receptor; treating, suppressing or altering the progression of a cancer.

31. The method of claim 30 wherein said immune response involves human carcinoma cells.

32. The method of claim 30, wherein the blocking agent is a modified inhibitory form of APRIL, or anti-APRIL antibodies or biologically active fragments thereof.

33. The method of Claim 32 wherein the blocking agent is an anti-APRIL receptor antibody.

34. The method of Claim 30 wherein the blocking agent is administered to a patient in combination with at least one chemotherapeutic agent.

35. The method of claim 34 further comprising the step of administering radiation therapy to said patient.

35. The use of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 for the preparation of a medicament for suppressing growth of a tumor cell that expresses APRIL, comprising the step of contacting said cell with an effective amount of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21.

36. The use of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 for the preparation of a medicament for suppressing growth of a tumor cell that expresses an APRIL receptor polypeptide, comprising the step of contacting said cell with an effective amount of a soluble APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 to said subject.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
A049PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/19191	11/09/1998	12/09/1997
International Patent Classification (IPC) or national classification and IPC C12N15/11		
Applicant BIOGEN, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09/04/1999	10.01.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx. 523656 epmu d Fax +49 89 2399 - 4465	Authorized officer Ury. A Telephone No +49 89 2399 8411

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/19191

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-33,40-45	as originally filed			

Claims, No.:

1-36	as received on	03/12/1999	with letter of	30/11/1999

Drawings, sheets:

1/6-6/6	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6-11, 14, 16-20, 23-25, 27-40
	No:	Claims	1-5, 12, 13, 15, 21, 22, 26
Inventive step (IS)	Yes:	Claims	6-11, 14, 16-20, 23-25, 27-40
	No:	Claims	1-5, 12, 13, 15, 21, 22, 26
Industrial applicability (IA)	Yes:	Claims	1-13, 15, 20-28
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Item I.

Almost all the claims (filed with the letter dated 30/11/99) have been amended. The amendments introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are *inter alia* the following:

- a polypeptide of at least about **102** amino acids and the nucleic acid encoding it (claims 1 and 12).
- a polypeptide comprising an amino acid sequence found in amino acids **1-55** of SEQ ID NO:2 and the nucleic acid encoding it (claims 3 and 13).
- a polypeptide comprising an amino acid sequence found in amino acids **157-250** of SEQ ID NO:2 and the nucleic acid encoding it (claims 4 and 14).

No basis for these amendments can be found in the application as filed (note that said amino acid numbers do not even appear *per se* in the original application). Hence the claims as amended result in the application being amended in such a way that it contains subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT.

Consequently, this report has been established as if said amendments had not been made (Rule 70.2(c) PCT), i.e. **has been established on the basis of claims 1-40 as originally filed.**

Item V.

Reference is made to the following document:

D1: Adams et al., EMBL DATABASE, 18/04/97, Heidelberg, DE, Accession number: AA361896.

I) Document D1 discloses a 303 bp EST corresponding to a T-cell lymphoma Homo sapiens cDNA 5' end. This cDNA encodes a 101 amino acid protein having 100% identity with a part of APRIL's amino acid sequence (i.e. amino acids 56 to 156).

The cDNA 5' end of D1 destroys the novelty (Article 33.2 PCT) of the following claims:
- claim 1 (due to the presence of the expression "a fragment thereof"),
- claims 2, 3 (due to the expression "consisting essentially" see item VIII.2),
- claims 4, 5 and 26.

The 101 amino acid protein disclosed in D1 destroys the novelty (Article 33.2 PCT) of the following claims:
- claim 12 (due to the expression "consisting essentially" see item VIII.2),
- claims 13 and 15 (the expression "a pharmaceutical composition" does not confer any distinguishing feature to the product and the formulation "a pharmaceutical acceptable carrier" is vague and can refer to water or buffers),
- claims 21 and 22b.

II) The claims which have not been objected to seem to meet the requirements of Article 33.2 and 3 PCT.

III) For the assessment of the present claims 14, 16-19, 29-40 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Item VI.

Certain published documents (Rule 70.10)

Application No	Publication date	Filing date
WO 97/33902	18.09.97	14.03.96

Item VIII.

1) The subject-matter of a great many claims do not fulfil the requirements of Article 6 PCT taken in conjunction with Rule 6.3a PCT which states that the subject-matter for which protection is sought must be defined in terms of the technical features of the invention.
The claims objected to are claims 1, 6, 8, 10, 11, 13, 14, 16-20, 23-25, 27-40.

2) The term "consisting essentially" used in many claims is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

3) Furthermore it should be noted that the words "hybridizing" and "hybridizes" (claims 4, 22b, 26) are not acceptable clear technical features per se, since the scope of protection defined by said features varies depending on the conditions used (Article 6 PCT).

What is claimed is:

1. Nucleic acid encoding an APRIL ligand, said ligand comprising a polypeptide of at least about 102 amino acids.

2. Nucleic acid of claim 1 encoding SEQ ID No:2.

3. Nucleic acid encoding an APRIL ligand or a fragment thereof, wherein said ligand comprises an amino acid sequence found in amino acids 1 to 55 of SEQ ID NO:2.

4. Nucleic acid encoding an APRIL ligand or a fragment thereof, wherein said ligand comprises an amino acid sequence found in amino acids 157 to 250 of SEQ ID NO:2.

5. Nucleic acid of claims 1-4 encoding an amino acid substitution analog of SEQ ID No:2.

6. Nucleic acid of claim 5 wherein said substitution analog, when aligned with SEQ ID No:2, shares at least 40% sequence similarity therewith, further wherein said substitution analog shares at least 80% of aligned cysteine residues with said APRIL ligand.

7. Nucleic acid of claim 5 wherein said substitution analog, when aligned with SEQ ID No:2, shares at least 80% sequence similarity therewith.

8. Nucleic acid having a nucleotide sequence comprising

(a) SEQ ID NO:1; or

(b) a substitution analog of SEQ ID NO:1; or

(c) an alteration analog of SEQ ID NO:1; or

(d) a deletion analog of SEQ ID NO:1.

9. A vector having the nucleic acid of claim 1, 2, 3, 4, or 8, present as an insert therein, said vector optionally comprising an expression control sequence operably linked to said insert.

10. A host cell comprising the vector of claim 9.

11. A method for producing substantially pure APRIL comprising the steps of: culturing the host cells of claim 10.

12. An APRIL ligand polypeptide comprising at least about 102 amino acids.

13. An APRIL ligand polypeptide comprising an amino acid sequence found in amino acids 1 to 55 of SEQ ID NO:2 or a fragment thereof.

14. An APRIL ligand polypeptide comprising an amino acid sequence found in amino acids 157 to 250 of SEQ ID NO:2 or a fragment thereof.

15. An APRIL ligand selected from:

(a) SEQ ID No:2; or

(b) an amino acid substitution analog of SEQ ID No:2.

16. A soluble APRIL ligand polypeptide of claims 12-15.

17. A pharmaceutical composition comprising a therapeutically effective amount of an APRIL ligand polypeptide of claims 12-15 and a pharmaceutically acceptable carrier.

19. An antibody that binds specifically to an APRIL ligand polypeptide of claim 12, 13, 14, 15 or 16.

20. An antibody that blocks binding of an APRIL ligand polypeptide to an APRIL receptor polypeptide.

21. An antibody of claim 20 that binds specifically to an APRIL polypeptide.

22. The use of a pharmaceutical composition of claim 17 for the preparation of a medicament for preventing or reducing the severity of an autoimmune disease; preventing or reducing the severity of an immune response to a tissue graft; stimulating or suppressing the immune system; or treating cancer.

23. A method for identifying a receptor for APRIL comprising:

a. providing APRIL or a fragment thereof,

b. labeling said APRIL or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled of step b.

24. A method of expressing APRIL in a mammalian cell comprising:

a. introducing a gene encoding APRIL into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

25. The use of a vector comprising a gene encoding APRIL for the preparation of a medicament for treating a disorder related to APRIL in a mammal comprising

a. introducing into a cell a therapeutically effective amount of said vector; and

b. expressing said gene in said mammalian cell.

26. The method of claim 25 wherein the mammal is a human.

27. The method of claim 25 wherein said vector is a virus.

28. The use of an agent capable of interfering with the binding of APRIL to a receptor for the preparation of a medicament for inducing cell death.

29. The method of claim 28 further comprising the administration of interferon-γ.

30. The use of a blocking agent capable of interfering with the association between APRIL and its receptor for the preparation of a medicament for treating, suppressing, activating or altering an immune response involving a signaling pathway between APRIL and its receptor; treating, suppressing or altering the progression of a cancer.

31. The method of claim 30 wherein said immune response involves human carcinoma cells.

32. The method of claim 30, wherein the blocking agent is a modified inhibitory form of APRIL, or anti-APRIL antibodies or biologically active fragments thereof.

33. The method of Claim 32 wherein the blocking agent is an anti-APRIL receptor antibody.

34. The method of Claim 30 wherein the blocking agent is administered to a patient in combination with at least one chemotherapeutic agent.

35. The method of claim 34 further comprising the step of administering radiation therapy to said patient.

35. The use of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 for the preparation of a medicament for suppressing growth of a tumor cell that expresses APRIL, comprising the step of contacting said cell with an effective amount of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21.

36. The use of an APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 for the preparation of a medicament for suppressing growth of a tumor cell that expresses an APRIL receptor polypeptide, comprising the step of contacting said cell with an effective amount of a soluble APRIL ligand polypeptide of claims 12-15 or an antibody of claims 19-21 to said subject.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

(76)

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, C07K 14/525, A61K 38/00	A3	(11) International Publication Number: WO 99/12965 (43) International Publication Date: 18 March 1999 (18.03.99)

(21) International Application Number: PCT/US98/19191

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60/058,786	12 September 1997 (12.09.97)	US
60/079,384	26 March 1998 (26.03.98)	US

(71) Applicant *(for all designated States except US)*: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: TSCHOPP, Jurg [CH/CH]; Chemin des Fontannins 10, CH–1066 Epalinges (CH).

(74) Agent: FLYNN, Kerry; Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
3 June 1999 (03.06.99)

(54) Title: APRIL– A NOVEL PROTEIN WITH GROWTH EFFECTS

(57) Abstract

APRIL, a novel member of the tumor necrosis factor family (TNF), modified APRILs, and pharmaceutical compositions comprising them.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 98/19191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K14/525 A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	HAHNE M. ET AL.: "APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 188, no. 6, 21 September 1998, pages 1185-1190, XP002099001 US see the whole document ---	1-40
X,P	WO 97 33902 A (HUMAN GENOME SCIENCES INC.) 18 September 1997 see the whole document --- -/--	1-15,17, 19-31, 34,36-40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1999	22/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 98/19191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ADAMS M.D. ET AL.: "EST71242 T-cell lymphoma Homo sapiens cDNA 5' end" EMBL DATABASE,18 April 1997, XP002099002 HEIDELBERG, DE Accession Number: AA361896	2-5,11, 12,15, 22,26
A	WO 96 40774 A (BIOGEN INC) 19 December 1996	14, 16-19, 32-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19191

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14, 16-19, 29-31, 34-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1 [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/19191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9733902 A	18-09-1997	AU 5366596 A	01-10-1997
		EP 0897390 A	24-02-1999
WO 9640774 A	19-12-1996	AU 6166396 A	30-12-1996

PCT/US98/19491

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

October 22, 1998

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/058,786*
FILING DATE: *September 12, 1997*
PCT APPLICATION NUMBER: *PCT/US98/19191*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. LAWRENCE
Certifying Officer

PTO/SB/16 (2-96)
Approved for use through 01/31/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (b)(2).

Docket Number	A034P	Type a plus sign (+) inside this box -->	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE NAME/INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Tschopp	Jurg		Epalinges, Switzerland

TITLE OF THE INVENTION (280 characters max)

NOVEL LIGANDS IN THE TNF FAMILY

CORRESPONDENCE ADDRESS (Including country if not United States)

Patent Administrator
Legal Department
Biogen, Inc.
14 Cambridge Center
Cambridge, MA 02142

ENCLOSED APPLICATION PARTS *(check all that apply)*

[X]	Specification	*Number of Pages*	44	[ ]	Small Entity Statement
[X]	Drawing(s)	*Number of Sheets*	1	[ ]	Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

[ ]	A check or money order is enclosed to cover the filing fees	FILING FEE AMOUNT ($)	$150.00
[X]	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-2327		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date 9/12/97

TYPED or PRINTED NAME Kerry A. Flynn

REGISTRATION NO. *(if appropriate)* 33,693

[ ] Additional inventors are being named on separately numbered sheets attached hereto

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Burden Hour Statment: This form is estimated to take .2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

APPLICATION

FOR

UNITED STATES PATENT

APPLICANTS: **Jurg Tschopp**

TITLE: **NOVEL LIGANDS IN THE TNF FAMILY**

"EXPRESS MAIL" mailing label number EM259501285US

Date of Deposit September 12, 1997

I hereby certify that this paper/fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Hon. Assistant Commissioner for Patents, Washington, D.C. 20231

Paula J. Hunt.

NOVEL LIGANDS IN THE TNF FAMILY

BACKGROUND OF THE INVENTION

The present invention relates to novel ligands, polypeptides which are members of the Tumor Necrosis Factor Family. These proteins or their receptors may have anti-cancer and/or immunoregulatory applications. Furthermore, cells transfected with the genes for these novel ligands may be used in gene therapy to treat tumors, autoimmune and inflammatory diseases or inherited genetic disorders, and blocking antitibodies to these proteins can have immunoregulatory applications.

BACKGROUND OF THE INVENTION

The tumor-necrosis factor (TNF)-related cytokines are mediators of host defense and immune regulation. Members of this family exist in membrane-anchored forms, acting locally through cell-to-cell contact, or as secreted proteins capable of diffusing to more distant targets. A parallel family of receptors signals the presence of these molecules leading to the initiation of cell death or cellular proliferation and differentiation in the target tissue. Presently, the TNF family of ligands and receptors has at least 11 recognized receptor-ligand pairs, including: TNF:TNF-R; LT-α:TNF-R; LT-α/β:LT-β-R; FasL:Fas; CD40L:CD40; CD30L:CD30; CD27L:CD27; OX40L:OX40 and 4-1BBL:4-1BB. The DNA sequences encoding these ligands have only about 25% to about 30% identity in even the most related cases, although the amino acid relatedness is about 50%.

The defining feature of this family of cytokine receptors is found in the cysteine rich extracellular domain initially revealed by the molecular cloning of two distinct TNF receptors.[1] This family of genes encodes glycoproteins characteristic of Type I transmembrane proteins with an extracellular ligand binding domain, a single membrane spanning region and a cytoplasmic region involved in activating cellular functions. The cysteine-rich ligand binding region exhibits a tightly knit disulfide linked core domain, which, depending upon the particular family member, is repeated multiple times. Most receptors have four domains, although there may be as few as three, or as many as six.

Proteins in the TNF family of ligands are characterized by a short N-terminal stretch of normally short hydrophilic amino acids, often containing several lysine or arginine residues

thought to serve as stop transfer sequences. Next follows a transmembrane region and an extracellular region of variable length, that separates the C-terminal receptor binding domain from the membrane. This region is sometimes referred to as the "stalk". The C-terminal binding region comprises the bulk of the protein, and often, but not always, contains glycosylation sites. These genes lack the classic signal sequences characteristic of type I membrane proteins, having type II membrane proteins with the C terminus lying outside the cell, and the short N-terminus residing in the cytoplasm. In some cases, e.g., TNF and LT-α, cleavage in the stalk region can occur early during protein processing and the ligand is then found primarily in secreted form. Most ligands, however, exist in a membrane form, mediating localized signaling.

The structure of these ligands has been well-defined by crystallographic analyses of TNF, LT-α, and CD40L. TNF and lymphotoxin-α (LT-α) are both structured into a sandwich of two anti-parallel β-pleated sheets with the "jelly roll" or Greek key topology.[ii] The rms deviation between the Cα and β-strand residues is 0.61 C, suggesting a high degree of similarity in their molecular topography. A structural feature emerging from molecular studies of CD40L, TNF and LT-α is the propensity to assemble into oligomeric complexes. Intrinsic to the oligomeric structure is the formation of the receptor binding site at the junction between the neighboring subunits creating a multivalent ligand. The quaternary structures of TNF, CD40L and LT-α have been shown to exist as trimers by analysis of their crystal structures. Many of the amino acids conserved between the different ligands are in stretches of the scaffold β-sheet. It is likely that the basic sandwich structure is preserved in all of these molecules, since portions of these scaffold sequences are conserved across the various family members. The quaternary structure may also be maintained since the subunit conformation is likely to remain similar.

TNF family members can best be described as master switches in the immune system controlling both cell survival and differentiation. Only TNF and LTα are currently recognized as secreted cytokines contrasting with the other predominantly membrane anchored members of the TNF family. While a membrane form of TNF has been well-characterized and is likely to have unique biological roles, secreted TNF functions as a general alarm signaling to cells more distant from the site of the triggering event. Thus TNF secretion can amplify an event leading to the well-described changes in the vasculature lining and the inflammatory state of cells. In contrast, the membrane bound members of the family send signals though the TNF type receptors only to

cells in direct contact. For example T cells provide CD40 mediated "help" only to those B cells brought into direct contact via cognate TCR interactions. Similar cell-cell contact limitations on the ability to induce cell death apply to the well-studied Fas system.

The ability to induce programmed cell death is an important and well-studied feature of several members of the TNF family. Fas mediated apoptosis appears to play a role in the regulation of autoreactive lymphocytes in the periphery and possibly the thymus (Castro et al., 1996) and recent work has also implicated the TNF and CD30 systems in the survival of T cells and large cell anaplastic lymphoma lines (Amakawa et al., 1996; Gruss et al., 1994; Sytwu et al., 1996; Zheng et al., 1995). We and others had previously shown the death of this line in response to TNF, Fas or LTb receptor signaling to have features of apoptosis (Abreu-Martin et al., 1995; Browning et al., 1996).

It appears that one can segregate the TNF ligands into three groups based on their ability to induce cell death (Table III). First, TNF, Fas ligand and TRAIL can efficiently induce cell death in many lines and their receptors mostly likely have good canonical death domains. Presumably the ligand to DR-3 (TRAMP/WSL-1) would also all into this category. Next there are those ligands which trigger a weaker death signal limited to few cell types and TRELL, CD30 ligand and LTa1b2 are examples of this class . How this group can trigger cell death in the absence of a canonical death domain is an interesting question and suggests that a separate weaker death signaling mechanism exists. Lastly, there are those members that cannot efficiently deliver a death signal. Probably all groups can have antiproliferative effects on some cell types consequent to inducing cell differentiation e.g. CD40 (Funakoshi et al., 1994)

The TNF family has grown dramatically in recent years to encompass at least 11 different signaling pathways involving regulation of the immune system. The expression patterns of TRELL and TRAIL indicate that there is still more functional variety to be uncovered in this family. This aspect has been especially highlighted in recent the discovery of two receptors that affect the ability of rous sacroma and herpes simplex virus to replicate as well as the historical observations that TNF has anti-viral activity and pox viruses encode for decoy TNF receptors (Brojatsch et al., 1996; Montgomery et al., 1996; Smith, 1994; Vassalli, 1992). The generation soluble TRELL and the identification of the TRELL receptor should provide the tools to elucidate the biological function of this interesting protein.

TNF is a mediator of septic shock and cachexia[iii], and is involved in the regulation of hematopoietic cell development.[iv] It appears to play a major role as a mediator of inflammation and defense against bacterial, viral and parasitic infections[v] as well as having antitumor activity.[vi] TNF is also involved in different autoimmune diseases.[vii] TNF may be produced by several types of cells, including macrophages, fibroblasts, T cells and natural killer cells.[viii] TNF binds to two different receptors, each acting through specific intracellular signaling molecules, thus resulting in different effects of TNF.[ix] TNF can exist either as a membrane bound form or as a soluble secreted cytokine.[x]

LT-α shares many activities with TNF, i.e. binding to the TNF receptors,[xi] but unlike TNF, appears to be secreted primarily by activated T cells and some β-lymphoblastoid tumors.[xii] The heteromeric complex of LT-α and LT-β is a membrane bound complex which binds to the LT-β receptor.[xiii] The LT system (LTs and LT-R) appears to be involved in the development of peripheral lymphoid organs since genetic disruption of LT-β leads to disorganization of T and B cells in the spleen and an absence of lymph nodes.[xiv] The LT-β system is also involved in cell death of some adenocarcinoma cell lines.[xv]

Fas-L, another member of the TNF family, is expressed predominantly on activated T cells.[xvi] It induces the death of cells bearing its receptor, including tumor cells and HIV-infected cells, by a mechanism known as programmed cell death or apoptosis.[xvii] Furthermore, deficiencies in either Fas or Fas-L may lead to lymphoproliferative disorders, confirming the role of the Fas system in the regulation of immune responses.[xviii] The Fas system is also involved in liver damage resulting from hepatitis chronic infection[xix] and in autoimmunity in HIV-infected patients.[xx] The Fas system is also involved in T-cell destruction in HIV patients.[xxi] TRAIL, another member of this family, also seems to be involved in the death of a wide variety of transformed cell lines of diverse origin.[xxii]

CD40-L, another member of the TNF family, is expressed on T cells and induces the regulation of CD40-bearing B cells.[xxiii] Furthermore, alterations in the CD40-L gene result in a disease known as X-linked hyper-IgM syndrome.[xxiv] The CD40 system is also involved in different autoimmune diseases[xxv] and CD40-L is known to have antiviral properties.[xxvi] Although the CD40 system is involved in the rescue of apoptotic B cells,[xxvii] in non-immune cells it induces apoptosis[xxviii]. Many additional lymphocyte members of the TNF family are also involved in costimulation.[xxix]

Generally, the members of the TNF family have fundamental regulatory roles in controlling the immune system and activating acute host defense systems. Given the current progress in manipulating members of the TNF family for therapeutic benefit, it is likely that members of this family may provide unique means to control disease. Some of the ligands of this family can directly induce the apoptotic death of many transformed cells e.g. LT, TNF, Fas ligand and TRAIL (Nagata, 1997). Fas and possibly TNF and CD30 receptor activation can induce cell death in nontransformed lymphocytes which may play an immunoregulatory function (Amakawa et al., 1996; Nagata, 1997; Sytwu et al., 1996; Zheng et al., 1995). In general, death is triggered following the aggregation of death domains which reside on the cytoplasmic side of the TNF receptors. The death domain orchestrates the assembly of various signal transduction components which result in the activation of the caspase cascade (Nagata, 1997). Some receptors lack canonical death domains, e.g. LTb receptor and CD30 (Browning et al., 1996; Lee et al., 1996) yet can induce cell death, albeit more weakly. It is likely that these receptors function primarily to induce cell differentiation and the death is an aberrant consequence in some transformed cell lines, although this picture is unclear as studies on the CD30 null mouse suggest a death role in negative selection in the thymus (Amakawa et al., 1996). Conversely, signaling through other pathways such as CD40 is required to maintain cell survival. Thus, there is a need to identify and characterize additional molecules which are members of the TNF family thereby providing additional means of controlling disease and manipulating the immune system.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to two novel polypeptides referred to as Tumor-ligand, and Kay-ligand, which substantially obviate one or more of the problems due to the limitations and disadvantages of the related art. The inventors have discovered new members of the TNF family of cytokines, and defined both the human amino acid sequence of the protein, as well as the DNA sequences encoding these proteins. The claimed invention may be used to identify new diagnostics and therapeutics for numerous diseases and conditions as discussed in more detail below, as well as to obtain information about, and manipulate, the immune system and its processes. Additionally, the invention may be involved in the induction of cell death in carcinomas.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent form the description, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof, as well as in the appended drawings.

Thus, to achieve these and other advantages, and in accordance with the purpose of the invention, as embodied and broadly described herein, the invention includes DNA sequences encoding Kay-ligand and Tumor-ligand Specifically, the invention relates to DNA sequences which encode the human Kay-ligand , SEQ. ID. NO.: 1, and the Tumor-ligand, SEQ. ID. NO. 5. Additionally, the claimed invention relates to the amino acid sequences of these novel ligands. The amino acid sequence of human Kay-ligand is set forth in SEQ. ID. NO.: 2, and the sequence for human Tumor-ligand is set forth in SEQ. ID. NO.: 6. Applicants have additionally provided in part the DNA sequence for murine Kay-ligand, SEQ. ID. NO.: 3, and the protein encoded by SEQ. ID. NO. 3 is provided in SEQ. ID. NO.: 4. In other embodiments, the invention relates to sequences that have at least 50% homology with DNA sequences encoding the C terminal receptor binding domain of the ligands and hybridize to the claimed DNA sequences or fragments thereof, and which encode the Kay- or Tumor - ligands having the sequences identified in SEQ. ID. NO. 1 or SEQ. ID. NO. 5.

The invention in certain embodiments furthermore relates to DNA sequences encoding Kay-ligand or Tumor-ligand where the sequences are operatively linked to an expression control sequence. Any suitable expression control sequences are useful in the claimed invention, and can easily be selected by one skilled in the art.

The invention also contemplates recombinant DNAs comprising a sequence encoding Kay-ligand, Tumor-ligand, or fragments thereof, as well as hosts with stably integrated Kay-ligand or Tumor-ligand sequences introduced into their genome, or possessing episomal elements. Any suitable host may be used in the invention, and can easily be selected by one skilled in the art without undue experimentation.

In other embodiments, the invention relates to methods of producing substantially pure Kay-ligands and/or Tumor-ligands comprising the step of culturing transformed hosts. In yet other embodiments, the invention relates to the Kay and/or Tumor-ligands essentially free of normally associated animal proteins.

The invention encompasses Kay-ligand and Tumor-ligand having the amino acid sequence identified in SEQ. ID. NO. 2 or SEQ. ID. NO. 6, respectively, as well as fragments or homologs thereof. In various embodiments, the amino acid and/or the DNA sequences may comprise conservative insertions, deletions and substitutions, as further defined below or may comprise fragments of said sequences.

The invention relates in other embodiments to soluble constructs comprising Kay-ligand or Tumor-ligand, or both, which may be used to directly trigger Kay-ligand or Tumor-ligand mediated pharmacological events. Such events may have useful therapeutic benefits in the treatment of cancer, tumors or the manipulation of the immune system to treat immunologic diseases. Soluble forms of the claimed ligands could be genetically reengineered to incorporate an easily recognizable tag, thereby facilitating the identification of the receptors for these ligands.

In yet other embodiments the invention relates to methods of gene therapy using the genes for Tumor-ligand or Kay-ligand, as disclosed and claimed herein.

The pharmaceutical preparations of the invention may, optionally, include pharmaceutically acceptable carriers, adjuvants, fillers, or other pharmaceutical compositions, and may be administered in any of the numerous forms or routes known in the art.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory, and are intended to provide further explanation of the invention as claimed.

The accompanying drawings are included to provide a further understanding of the invention, and are incorporated in, and constitute a part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Northern Blot of Tumor Ligand in various tissues.

DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention. This invention relates to DNA sequences that code for human or mouse Kay-ligands

or Tumor-ligands, fragments and homologs thereof, and expression of those DNA sequences in hosts transformed with them. The invention relates to uses of these DNA sequences and the peptides encoded by them. Additionally, the invention encompasses both human and mouse amino acid sequences for Kay-ligand and Tumor-ligand, or fragments thereof, as well as pharmaceutical compositions comprising or derived from them.

A. DEFINITIONS

"Homologous", as used herein, refers to the sequence similarity between sequences of molecules being compared. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

A "purified preparation" or a "substantially pure preparation" of a polypeptide, as used herein, means a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from other substances, e.g., antibodies, matrices, etc., which are used to purify it.

"Transformed host" as used herein is meant to encompass any host with stably integrated sequence, i.e. Kay-ligand or Tumor-ligand sequence, introduced into its genome or a host possessing sequence, i.e. Ligand encoding episomal elements.

A "treatment", as used herein, includes any therapeutic treatment, e.g., the administration of a therapeutic agent or substance, e.g., a drug.

A "substantially pure nucleic acid", e.g., a substantially pure DNA, is a nucleic acid which is one or both of: (1) not immediately contiguous with either one or both of the sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or (2) which is substantially free of a nucleic acid sequence with which it occurs in the

organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding Tumor-ligand or Kay-ligand.

The terms "peptides", "proteins", and "polypeptides" are used interchangeably herein.

"Biologically active" as used herein, means having an in vivo or in vitro activity which may be performed directly or indirectly. Biologically active fragments of Tumor-ligand or Kay ligand may have, for example, 70% amino acid homology with the active site of the Ligands, more preferably at least 80%, and most preferably, at least 90% homology. Identity or homology with respect to the Ligands is defined herein as the percentage of amino acid residues in the candidate sequence which are identical to the Tumor-ligand or Kay-ligand residues in SEQ. ID. NOS. 6 or 2.

"Ligand" as used herein generically refers to either the Kay-ligand, the Tumor-ligand, or both. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature.

B. DNA SEQUENCES OF THE INVENTION

As described herein, one aspect of the invention features a substantially pure (or recombinant) nucleic acid which includes a nucleotide sequence encoding a Kay-ligand or Tumor-ligand, such as the DNA described in SEQ. ID. NOs. 1 or 5 and/or equivalents of such nucleic acids. The term nucleic acid as used herein can include fragments and equivalents, such as, for example, sequences encoding functionally equivalent peptides. Equivalent nucleotide sequences may include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, mutations, etc. and include sequences that differ from the nucleotide sequence encoding Kay-ligand or Tumor-ligand shown in SEQ. ID NO: 1 or 5, due to the degeneracy of the genetic code.

The inventor describes herein the human and sequences; the invention will be described generally by reference to the human sequences, although one skilled in the art will understand that the mouse sequences are encompassed herein. The human proteins appear to have all of the characteristics of the TNF family, i.e., a type II membrane protein organization and conservation of the sequence motifs involved in the folding of the protein into the TNF anti-parallel β-sheet structure.

The Tumor -L sequence encodes the entire protein, but the Kay-L sequence is partial, and lacks the complete 5' end. The complete sequence can easily be obtained by one skilled in the art by library screening, RACE, methods, primer extension or genomic sequencing. The nucleotide sequence for Kay-ligand is set forth in SEQ. ID. NO. 1 ; the amino acid sequence for Kay-ligand is described in SEQ. ID. NO. 2. The DNA and amino acid sequences for Tumor-ligand are described in SEQ. ID. NOS. 5 and 6 respectively.

The sequences of the invention can be used to prepare a series of DNA probes that are useful in screening various collections of natural and synthetic DNAs for the presence of DNA sequences that code for Kay-ligand , Tumor-ligand, or fragments or derivatives thereof. One skilled in the art will recognize that reference to Kay-ligand, Tumor-ligand, or Ligands as used herein, refers also to biologically active derivatives, fragments or homologs thereof.

The DNA sequences encoding the Ligands of the invention can be employed to produce the claimed peptides on expression in various prokaryotic and eukaryotic hosts transformed with them. These peptides may be used in anti-cancer, and immunoregulatory applications. In general, this comprises the steps of culturing a host transformed with a DNA molecule

containing the sequence encoding one of the Ligands, operatively-linked to an expression control sequence.

The DNA sequences and recombinant DNA molecules of the present invention can be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal or synthetic DNA sequences. The expression vectors of the invention are characterized by at least one expression control sequence that may be operatively linked to one of the Ligand DNA sequence inserted in the vector, in order to control and to regulate the expression of the DNA sequence.

Furthermore, within each expression vector, various sites may be selected for insertion of a Ligand sequence of the invention. The sites are usually designated by a restriction endonuclease which cuts them, and these sites and endonucleases are well recognized by those skilled in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the desired DNA fragment. Instead, the vector may be cloned to the fragment by alternate means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment, and its operative linking therein to an expression control sequence, is determined by a variety of factors. These factors include, but are not limited to, the size of the protein to be expressed, the susceptibility of the desired protein to proteolytic degradation by host cell enzymes, number of sites susceptible to a particular restriction enzyme, contamination or binding of the protein to be expressed by host cell proteins which may prove difficult to remove during purification. Additional factors which may be considered include expression characteristics such as the location of start and stop codons relative to the vector sequences, and other factors which will be recognized by those skilled in the art. The choice of a vector and insertion site for the claimed DNA sequences is determined by a balancing of these factors, not all selections being equally effective for a desired application. However, it is routine for one skilled in the art to analyze these parameters and choose an appropriate system depending on the particular application.

One skilled in the art can readily make appropriate modifications to the expression control sequences to obtain higher levels of protein expression, i.e. by substitution of codons, or selecting codons for particular amino acids that are preferentially used by particular organisms, to minimize proteolysis or to alter glycosylation composition. Likewise, cysteines may be changed to other amino acids to simplify production, refolding or stability problems.

Thus, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention. However, a particular selection of a host/expression vector combination may be made by those of skill in the art. Factors one may consider include, for example, the compatibility of the host and vector, toxicity to the host of the proteins encoded by the DNA sequence, ease of recovery of the desired protein, expression characteristics of the DNA sequences and expression control sequences operatively linked to them, biosafety, costs and the folding, form or other necessary post-expression modifications of the desired protein.

The Kay-ligand, Tumor-ligand and homologs thereof produced by hosts transformed with the sequences of the invention, as well as native Ligands purified by the processes of this invention, or produced from the claimed amino acid sequences, are useful in a variety of compositions and methods for anticancer, antitumor and immunoregulatory applications. They are also useful in therapy and methods directed to other diseases.

This invention also relates to the use of the DNA sequences disclosed herein to express these Ligands under abnormal conditions, i.e. in a gene therapy setting. Kay-ligand or Tumor-ligand may be expressed in tumor cells under the direction of promoters appropriate for such applications. Such expression could enhance anti-tumor immune responses or directly affect the survival of the tumor. The claimed Ligands can also affect the survival of an organ graft by altering the local immune response. In this case, the graft itself or the surrounding cells would be modified with an engineered gene encoding Kay-ligand or Tumor-ligand.

Another aspect of the invention relates to the use of the isolated nucleic acid encoding either the Kay-ligand or the Tumor-ligand in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular conditions with the cellular mRNA and/or DNA encoding the Ligand of interest, so as to inhibit expression of the encoded protein, i.e. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to a range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA which is complementary to at least a portion of the cellular mRNA which encodes Kay-ligand or Tumor-ligand. Alternatively, the antisense construct can be an oligonucleotide probe which is generated ex vivo. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, and are therefor stable in vivo. Exemplary nucleic acids molecules for use as antisense oligonucleotides are phosphoramidates, phosphothioate and methylphosphonate analogs of DNA (See, e.g., 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van Der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48: 2659-2668, specifically incorporated herein by reference.

C. KAY-LIGAND, TUMOR-LIGAND AND AMINO ACID SEQUENCES THEREFOR

The Ligands of the invention, as discussed above, are members of the TNF family. The protein, fragments or homologs thereof may have wide therapeutic and diagnostic applications.

The Kay-ligand is present primarily in the spleen and in peripheral blood lymphocytes, strongly indicating a regulatory role in the immune system. The Tumor-ligand is weakly expressed in these same organs but is abundantly found in many tumor lines. The upregulation in tumors suggests a role in tumor formation and/or survival, and exactly such roles have been found for the expression of FAS-L in solid tumors. Hahne, M. et al. (1996).

Although the precise three dimensional structure of the claimed Ligands is not known, it is predicted that, as members of the TNF family, they may share certain structural characteristics with other members of the family.

Comparison of the claimed Tumor-ligand and Kay-ligand sequences with other members of the human TNF family reveals considerable structural similarity. All the proteins share several regions of sequence conservation in the extracellular domain.

The novel polypeptides of the invention specifically interact with a receptor, which has not yet been identified. However, the peptides and methods disclosed herein enable the

identification of receptors which specifically interact with the claimed Ligands or fragments thereof.

The claimed invention in certain embodiments includes peptides derived from Tumor-ligand or Kay-ligand which have the ability to bind to their receptors. Fragments of these Ligands can be produced in several ways, e.g., recombinantly, by PCR, proteolytic digestion or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end or both ends of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNAs which encode a variety of fragments. DNAs which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above discussed methods.

Polypeptide fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f- moc or t-boc chemistry. For example, peptides and DNA sequences of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragment, or divided into overlapping fragments of a desired length. Methods such as these are described in more detail below.

D. Generation of Soluble Forms of Kay-ligand and Tumor-ligand

Soluble forms of the ligand can often signal effectively and hence can be administered as a drug which now mimics the natural membrane form. It is possible that the Ligands claimed herein are naturally secreted as soluble cytokines, however, if not, one can reengineer the gene to force secretion. To create a soluble secreted form of either Kay-ligand or Tumor-Ligand, one would remove at the DNA level the N-terminus transmembrane regions, and some portion of the stalk region, and replace them with a type I leader or alternatively a type II leader sequence that will allow efficient proteolytic cleavage in the chosen expression system. A skilled artisan could vary the amount of the stalk region retained in the secretion expression construct to optimize both receptor binding properties and secretion efficiency. For example, the constructs containing all possible stalk lengths, i.e. N-terminal truncations, could be prepared such that proteins starting at amino acids 81 to 139 would result. The optimal length stalk sequence would result from this type of analysis.

E. Generation of Antibodies Reactive with the Kay-ligand or Tumor-ligand

The invention also includes antibodies specifically reactive with the claimed Ligands or their receptors. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers, or other techniques, well known in the art.

An immunogenic portion of the claimed Ligands or their receptors can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of Kay-ligand, Tumor-ligand, or their receptors, e.g. antigenic determinants of a polypeptide of SEQ. ID. NO.: 2 or 6 , or a closely related human or non-human mammalian homolog (e.g. 70, 80 or 90 percent homologous, more preferably at least 95 percent homologous). In yet a further preferred embodiment of the present invention, the anti-Ligand or anti-Ligand-receptor antibodies do not substantially cross react (i.e. react specifically) with a protein which is e.g., less than 80 percent homologous to SEQ. ID. NO. 2 or 6; preferably less than 90 percent homologous with SEQ. ID. NO.: 2 or 6; and, most preferably less than 95 percent homologous with SEQ. ID. NO.: 2 or 6. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is less than 10 percent, more preferably less than 5 percent, and even more preferably less than 1 percent, of the binding affinity for a protein of SEQ. ID. NO. 2 or 6.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with Kay-ligand, Tumor-ligand, or their receptors. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibodies of the present invention are further intended to include biospecific and chimeric molecules having anti-Ligand or anti-Ligand -receptor activity. Thus, both monoclonal and polyclonal antibodies (Ab) directed against Kay-

ligand, Tumor-ligand and their receptors, and antibody fragments such as Fab` and $F(ab`)_2$, can be used to block the action of the Ligand and their respective receptor.

Various forms of antibodies can also be made using standard recombinant DNA techniques. (Winter and Milstein, Nature 349: 293-299 (1991) specifically incorporated by reference herein.) For example, chimeric antibodies can be constructed in which the antigen binding domain from an animal antibody is linked to a human constant domain (e.g. Cabilly et al., U.S. 4,816,567, incorporated herein by reference). Chimeric antibodies may reduce the observed immunogenic responses elicited by animal antibodies when used in human clinical treatments.

In addition, recombinant "humanized antibodies" which recognize Tumor-ligand, Kay-ligand or their receptors can be synthesized. Humanized antibodies are chimeras comprising mostly human IgG sequences into which the regions responsible for specific antigen-binding have been inserted. Animals are immunized with the desired antigen, the corresponding antibodies are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized antibodies minimize the use of heterologous (i.e. inter species) sequences in human antibodies, and thus are less likely to elicit immune responses in the treated subject.

Construction of different classes of recombinant antibodies can also be accomplished by making chimeric or humanized antibodies comprising variable domains and human constant domains (CH1, CH2, CH3) isolated from different classes of immunoglobulins. For example, antibodies with increased antigen binding site valencies can be recombinantly produced by cloning the antigen binding site into vectors carrying the human : chain constant regions. (Arulanandam et al., J. Exp. Med., 177: 1439-1450 (1993), incorporated herein by reference.)

In addition, standard recombinant DNA techniques can be used to alter the binding affinities of recombinant antibodies with their antigens by altering amino acid residues in the vicinity of the antigen binding sites. The antigen binding affinity of a humanized antibody can be increased by mutagenesis based on molecular modeling. (Queen et al., Proc. Natl. Acad. Sci. 86: 10029-33 (1989) incorporated herein by reference.

F. Generation of Analogs: Production of Altered DNA and Peptide Sequences

Analogs of the claimed Ligands can differ from the naturally occurring Ligands in amino acid sequence, or in ways that do not involve sequence, or both. Non-sequence modifications include in vivo or in vitro chemical derivatization of the Ligands. Non-sequence modifications include, but are not limited to, changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

Preferred analogs include Kay-ligand, Tumor-ligand or biologically active fragments thereof, whose sequences differ from the sequence given in SEQ. ID NOS. 2 and 6, by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the activity of Kay-ligand or Tumor-ligand. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g. substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and, phenylalanine, tyrosine.

TABLE 1

CONSERVATIVE AMINO ACID REPLACEMENTS

for amino Acid	code	replace with any of:
Alanine	A	D-Ala, Gly, Beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met

Lysine	K	D-Lys, Arg, D-Arg, Homo-arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3, 4 or 5-phenylproline, cis-3, 4, or 5-phenylproline
Proline	P	D-Pro, L-I-thoazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Useful methods for mutagenesis include PCR mutagenesis and saturation mutagenesis as discussed in more detail below. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences.

-PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity can be used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized can be amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments can be inserted into appropriate cloning vectors to provide random mutant libraries.

-Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro,* and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as of a protein can be prepared by random mutagenesis of DNA which those that alter function, can be obtained. The distribution of point mutations is not biased toward conserved sequence elements.

-Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art[xxx] Such techniques have been employed in the directed evolution of other proteins[xxxi].

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid

sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

-Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989) specifically incorporated by reference. In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions can then be refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

-Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983) incorporated herein by reference. Briefly, the desired DNA can be altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize

properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]) incorporated herein by reference.

-Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]) incorporated herein by reference. The starting material can be a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

-Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into

replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, binding to Kay-ligand, Tumor-ligand or their receptors, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

The invention also provides for reduction of the protein binding domains of the claimed polypeptides or their receptors, to generate mimetics, e.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of Kay-ligand or Tumor-ligand with their respective receptor. The critical residues of the Ligand involved in molecular recognition of a receptor polypeptide or of a downstream intracellular protein, can be determined and used to generate the Ligand or its receptor-derived peptidomimetics which competitively or noncompetitively inhibit binding of the Ligand with a receptor. (see, for example, "Peptide inhibitors of human papilloma virus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A), specifically incorporated herein by reference.

G. PHARMACEUTICAL COMPOSITIONS

By making available purified and recombinant- Kay-ligands and Tumor-ligands, the present invention provides assays which can be used to screen for drug candidates which are either agonists or antagonists of the normal cellular function, in this case, of Kay-ligand, Tumor-ligand or their receptors. In one embodiment, the assay evaluates the ability of a compound to modulate binding between the Ligands and their receptors. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by the skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular

target as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

Pharmaceutical compositions of the invention may comprise a therapeutically effective amount of Kay-ligand, Tumor-ligand, or their receptors, or fragments or mimetics thereof, and, optionally may include pharmaceutically acceptable carriers. Accordingly, this invention provides methods for treatment of cancer, and methods of stimulating, or in certain instances, inhibiting the immune system, or parts thereof by administering a pharmaceutically effective amount of a compound of the invention or its pharmaceutically acceptable salts or derivatives. It should of course by understood that the compositions and methods of this invention can be used in combination with other therapies for various treatments.

The compositions can be formulated for a variety of routes of administration, including systemic, topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the compositions of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compositions may be formulated in solid form and, optionally, redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

The compositions can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration, bile salts, fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the compositions are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams as known in the art.

Preferably the compositions of the invention will be in the form of a unit dose and will be administered one or more times a day. The amount of active compound administered at one time or over the course of treatment will depend on many factors. For example, the age and size of the subject, the severity and course of the disease being treated, the manner and form of administration, and the judgments of the treating physician. However, an effective dose may be

in the range of from about 0.005 to about 5 mg/kg/day, preferably about 0.05 to about 0.5 mg/kg/day. One skilled in the art will recognize that lower and higher doses may also be useful.

Gene constructs according to the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a Tumor-ligand or Kay-ligand polypeptide.

Expression constructs of the claimed Ligands can be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the gene for the claimed Ligands to cells in vivo. Approaches include insertion of the gene in viral vectors which can transfect cells directly, or delivering plasmid DNA with the help of, for example, liposomes, or intracellular carriers, as well as direct injection of the gene construct. Viral vector transfer methods are preferred.

A pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA, RNA or amino acid sequences to which they specifically bind. In other aspects, the claimed invention may be used to evaluate a chemical entity for its ability to interact with, e.g., bind or physically associate with a claimed Ligand, or fragment thereof. The method includes contacting the chemical entity with the Ligand, and evaluating the ability of the entity to interact with the Ligand. Additionally, the Ligands of the invention can be used in methods of evaluating naturally occurring ligands or receptors of these Ligands, as well as to evaluate chemical entities which associate or bind with receptors of the Ligands.

In certain aspects, the claimed invention features a method for evaluating a chemical entity for the ability to modulate the interaction between Kay-ligand, Tumor-ligand and their respective receptors. The method includes combining a Ligand receptor, and the Ligand under conditions wherein the pair is capable of interacting, adding the chemical entity to be evaluated and detecting the formation or dissolution of complexes. These modulating agents may be

further evaluated in vitro, e.g. by testing its activity in a cell free system, and then, optionally administering the compound to a cell or animal, and evaluating the effect.

H. EXAMPLES

c) Isolation of a receptor binding to the claimed Ligands

Ligands of the TNF family can be used to identify and clone receptors. With the described Kay-ligand and Tumor-ligand sequences, one could fuse the 5' end of the extracellular domain of these Ligands which constitutes the receptor binding sequence to a marker or tagging sequence and then add a leader sequence that will force secretion of the Ligand in any of a number of expression systems. One example of this technology is described by Browning et al., (1996) (JBC 271, 8618-8626) where the LT-β ligand was secreted in such a form. The VCAM leader sequence was coupled to a short myc peptide tag followed by the extracellular domain of the LT-β. The VCAM sequence is used to force secretion of the normally membrane bound LT-β molecule. The secreted protein retains a myc tag on the N-terminus which does not impair the ability to bind to a receptor. Such a secreted protein can be expressed in either transiently transfected Cos cells or a similar system, e.g., EBNA derived vectors, insect cell/baculovirus, picchia etc. The unpurified cell supernatant can be used as a source of the tagged ligand.

Cells expressing the receptor can be identified by exposing them to the tagged ligand. Cells with bound ligand are identified in a FACS experiment by labeling the myc tag with an anti-myc peptide antibody (9E10) followed by phycoerythrin (or a similar label) labeled anti-mouse immunoglobulin. FACS positive cells can be readily identified and would serve as a source of RNA encoding for the receptor. An expression library would then be prepared from this RNA via standard techniques and separated into pools. Pools of clones would be transfected into a suitable host cell and binding of the tagged ligand to receptor positive transfected cells determined via microscopic examination, following labeling of bound myc peptide tag with an enzyme labeled anti-mouse Ig reagent, i.e. galactosidase, alkaline phosphatase or luciferase labeled antibody. Once a positive pool has been identified, the pool size would be reduced until the receptor encoding cDNA is identified. This procedure could be carried out with either the mouse of human Kay-ligand or Tumor-ligand, as one may more readily lead to a receptor.

It will be apparent to those skilled in the art that various modifications and variations can be made in the novel Ligands, compositions and methods of the present invention without

departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

SEQ ID NO:1

```
      1  GCCGCCCTGC AAGGGGACCT GGCCAGCCTC CGGGCAGAGC TGCAGGGCCA
     51  CCACGCGGAG AAGCTGCCAG CAGGAGCAGG AGCCCCCAAG GCCGGCCTGG
    101  AGGAAGCTCC AGCTGTCACC GCGGGACTGA AAATCTTTGA ACCACCAGCT
    151  CCAGGAGAAG GCAACTCCAG TCAGAACAGC AGAAATAAGC GTGCCGTTCA
    201  GGGTCCAGAA GAAACAGTCA CTCAAGACTG CTTGCAACTG ATTGCAGACA
    251  GTGAAACACC AACTATACAA AAAGGATCTT ACACATTTGT TCCATGGCTT
    301  CTCAGCTTTA AAAGGGGAAG TGCCCTAGAA GAAAAAGAGA ATAAAATATT
    351  GGTCAAAGAA ACTGGTTACT TTTTTATATA TGGTCAGGTT TTATATACT
    401  ATAAGACCTA CGCCATGGGA CATCTAATTC AGAGGAAGAA GGTCCATGTC
    451  TTTGGGGATG AATTGAGTCT GGTGACTTTG TTTCGATGTA TTCAAAATAT
    501  GCCTGAAACA CTACCCAATA ATTCCTGCTA TTCAGCTGGC ATTGCAAAAC
    551  TGGAAGAAGG AGATGAACTC CAACTTGCAA TACCAAGAGA AAATGCACAA
    601  ATATCACTGG ATGGAGATGT CACATTTTTT GGTGCATTGA AACTGCTGTG
    651  ACCTACTTAC ACCATGTCTG TAGCTATTTT CCTCCCTTTC TCTGTACCTC
    701  TAAGAAGAAA GAATCTAACT GAAAATA
```

SEQ ID NO:2

```
      1  AALQGDLASL RAELQGHHAE KLPAGAGAPK AGLEEAPAVT AGLKIFEPPA
     51  PGEGNSSQNS RNKRAVQGPE ETVTQDCLQL IADSETPTIQ KGSYTFVPWL
    101  LSFKRGSALE EKENKILVKE TGYFFIYGQV LYTDKTYAMG HLIQRKKVHV
    151  FGDELSLVTL FRCIQNMPET LPNNSCYSAG IAKLEEGDEL QLAIPRENAQ
    201  ISLDGDVTFF GALKLL
```

SEQ ID NO:3

```
      1  TCTCAGCTCC TCCTGCACCA TGCCTGCCTG GATGCCGCCA TTCTCAACAT
     51  GATGATAATG GAATGAACCT CAGAAACAGA ACTTACACAT TTGTTCCATG
    101  GCTTCTCAGC TTTAAAAGAG GAAATGCCTT GGAGGAGAAA GAGAACAAAA
    151  TAGTGGTGAG GCAAACAGGC TATTTCTTCA TCTACAGCCA GGTTCTATAC
    201  ACGGACCCCA TCTTTGCTAT GGGTCATGTC ATCCAGAGGA AGAAAGTACA
    251  CGTCTTTGGG GACGAGCTGA GCCTGGTGAC CCTGTTCCGA TGTATTCAGA
    301  ATATGCCCAA AACACTGCCC AACAATTCCT GCTACTCGGC TGGCATCGCG
    351  AGGCTGGAAG AAGGAGATGA GATTCAGCTT GCAATTCCTC GGGAGAATGC
    401  ACAGATTTCA CGCAACGGAG ACGACACCTT CTTTGGTGCC CTAAAACTGC
    451  TGTAACTCAC TTGCTGGAGT GCGTGATCCC CTTCCCTCGT CTTCTCTGTA
    501  CCTCCGAGGG AGAAACAGAC GACTGGAAAA ACTAAAAGAT GGGGAAAGCC
    551  GTCAGCGAAA GTTTTCTCGT GACCCGTTGA ATCTGATCCA AACCAGGAAA
    601  TATAACAGAC AGCCACA
```

SEQ ID NO:4

```
YTF VPWLLSFKRG NALEEKENKI
VVRQTGYFFI YSQVLYTDPI FAMGHVIQRK KVHVFGDELS LVTLFRCIQN
MPKTLPNNSC YSAGIARLEE GDEIQLAIPR ENAQISRNGD DTFFGALKLL
```

SEQ ID NO:5

```
   1  GGTACGAGGC TTCCTAGAGG GACTGGAACC TAATTCTCCT GAGGCTGAGG
  51  GAGGGTGGAG GGTCTCAAGG CAACGCTGGC CCCACGACGG AGTGCCAGGA
 101  GCACTAACAG TACCCTTAGC TTGCTTTCCT CCTCCCTCCT TTTTATTTTC
 151  AAGTTCCTTT TTATTTCTCC TTGCGTAACA ACCTTCTTCC CTTCTGCACC
 201  ACTGCCCGTA CCCTTACCCG CCCCGCCACC TCCTTGCTAC CCCACTCTTG
 251  AAACCACAGC TGTTGGCAGG GTCCCCAGCT CATGCCAGCC TCATCTCCTT
 301  TCTTGCTAGC CCCCAAAGGG CCTCCAGGCA ACATGGGGGG CCCAGTCAGA
 351  GAGCCGGCAC TCTCAGTTGC CCTCTGGTTG AGTTGGGGGG CAGCTCTGGG
 401  GGCCGTGGCT TGTGCCATGG CTCTGCTGAC CCAACAAACA GAGCTGCAGA
 451  GCCTCAGGAG AGAGGTGAGC CGGCTGCAGG GGACAGGAGG CCCCTCCCAG
 501  AATGGGGAAG GGTATCCCTG GCAGAGTCTC CCGGAGCAGA GTTCCGATGC
 551  CCTGGAAGCC TGGGAGAATG GGGAGAGATC CCGGAAAAGG AGAGCAGTGC
 601  TCACCCAAAA ACAGAAGAAG CAGCACTCTG TCCTGCACCT GGTTCCCATT
 651  AACGCCACCT CCAAGGATGA CTCCGATGTG ACAGAGGTGA TGTGGCAACC
 701  AGCTCTTAGG CGTGGGAGAG GCCTACAGGC CCAAGGATAT GGTGTCCGAA
 751  TCCAGGATGC TGGAGTTTAT CTGCTGTATA GCCAGGTCCT GTTTCAAGAC
 801  GTGACTTTCA CCATGGGTCA GGTGGTGTCT CGAGAAGGCC AAGGAAGGCA
 851  GGAGACTCTA TTCCGATGTA TAAGAAGTAT GCCCTCCCAC CCGGACCGGG
 901  CCTACAACAG CTGCTATAGC GCAGGTGTCT TCCATTTACA CCAAGGGGAT
 951  ATTCTGAGTG TCATAATTCC CCGGGCAAGG GCGAAACTTA ACCTCTCTCC
1001  ACATGGAACC TTCCTGGGGT TTGTGAAACT GTGATTGTGT TATAAAAAGT
1051  GGCTCCCAGC TTGGAAGACC AGGGTGGGTA CATACTGGAG ACAGCCAAGA
1101  GCTGAGTATA TAAAGGAGAG GGAATGTGCA GGAACAGAGG CATCTTCCTG
1151  GGTTTGGCTC CCCGTTCCTC ACTTTTCCCT TTTCATTCCC ACCCCCTAGA
1201  CTTTGATTTT ACGGATATCT TGCTTCTGTT CCCCATGGAG CTCCGAATTC
1251  TTGCGTGTGT GTAGATGAGG GGCGGGGGAC GGGCGCCAGG CATTGTTCAG
1301  ACCTGGTCGG GGCCCACTGG AAGCATCCAG AACAGCACCA CCATCTTA
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SEQ ID NO:6

```
   1  MPASSPFLLA PKGPPGNMGG PVREPALSVA LWLSWGAALG AVACAMALLT
  51  QQTELQSLRR EVSRLQGTGG PSQNGEGYPW QSLPEQSSDA LEAWENGERS
 101  RKRRAVLTQK QKKQHSVLHL VPINATSKDD SDVTEVMWQP ALRRGRGLQA
 151  QGYGVRIQDA GVYLLYSQVL FQDVTFTMGQ VVSREGQGRQ ETLFRCIRSM
 201  PSHPDRAYNS CYSAGVFHLH QGDILSVIIP RARAKLNLSP HGTFLGFVKL
```

I claim:

1. A DNA sequence encoding Kay-ligand or a fragment thereof.

2. A DNA sequence encoding Kay-ligand, said sequence consisting essentially of SEQ. ID. NO. 1 or SEQ. ID. NO. 3.

3. A DNA sequence consisting essentially of SEQ. ID. NO. 1 or SEQ. ID. NO. 3, said DNA encoding a polypeptide, said polypeptide consisting essentially of SEQ. ID. NO. 2 or SEQ. ID. NO. 4.

4. A DNA sequence that hybridizes to at least a fragment of SEQ. ID NO. 1 or SEQ. ID NO. 3 said fragment comprising at least 20 consecutive bases, said DNA sequence encoding a polypeptide that is at least 30% homologous with an active site of Kay-ligand.

5. A DNA sequence according to claim 2 wherein said sequence consists essentially of SEQ. ID. NO. 1 or SEQ. ID. NO. 3 with conservative substitutions, alterations or deletions.

6. A recombinant DNA molecule comprising a DNA sequence encoding Kay-ligand, said sequence operatively linked to an expression control sequence.

7. The molecule of claim 6 comprising SEQ. ID. NO. 1 or SEQ. ID. NO. 3.

8. A unicellular host transformed with a recombinant DNA molecule of claim 6 or 7.

9. A DNA sequence encoding Kay-ligand having the amino acid sequence of SEQ. ID. NO. 2 or SEQ. ID. NO 4.

10. A method for producing substantially pure Kay-ligand comprising the step of culturing the unicellular host of claim 8.

11. Kay-ligand essentially free of normally associated animal proteins.

12. The Kay-ligand of claim 11 consisting essentially of SEQ. ID. NO. 2 or SEQ. ID. NO. 4.

13. A pharmaceutical composition comprising a therapeutically effective amount of Kay-ligand or an active fragment thereof, and a pharmaceutically acceptable carrier.

14. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

15. The pharmaceutical composition of claim 13 wherein said Kay-ligand or active fragment thereof comprises SEQ. ID. NO. 2 , or SEQ. ID. NO. 4, or a biologically active fragment thereof.

16. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

17. A method for stimulating the immune system comprising administering the composition of claim 13.

18. A method for suppressing the immune system comprising administering an effective amount of the pharmaceutical composition according to claim 13.

19. A method for treating cancer comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 13.

20. A method for identifying a receptor for the Kay-ligand comprising:

a. providing the Kay-ligand or a fragment thereof,

b. labeling said Kay-ligand or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled Kay-ligand of step b.

21. A soluble biologically active fragment of the Kay-ligand of claim 11.

22. A polypeptide comprising an amino acid sequence that is encoded by a DNA selected from the group consisting of:

a. a DNA sequence comprising SEQ. ID. NO. 1 or SEQ. ID. NO. 3;

b. a DNA sequence that hybridizes to the DNA defined in a. and coding on expression for a polypeptide that is at least 40% homologous with the Kay-ligand of claim 12.

23. An antibody preparation that is reactive to Kay-ligand or its receptor or biologically active fragments thereof.

24. The antibody preparation of claim 23 comprising monoclonal antibodies.

25. A method for producing an antibody preparation reactive to Kay-ligand or its receptor comprising the step of immunizing an organism with Kay-ligand or its receptor, or an antigenic fragment thereof.

26. An antisense nucleic acid against Kay-ligand comprising a nucleic acid sequence hybridizing to at least a portion of SEQ. ID. NO. 1 or SEQ. ID. NO. 3.

27. A pharmaceutical composition comprising an antibody preparation according to claim 24.

28. A method of expressing a gene in a mammalian cell comprising:

a. introducing a gene encoding Kay-ligand into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

29. A method of treating a disorder related to Kay-ligand in a mammal

a. introducing into a cell a therapeutically effective amount of a vector comprising a gene encoding the Kay-ligand; and

b. expressing said gene in said mammalian cell.

30. The method of claim 29 wherein the mammal is a human.

31. The method of claim 29 wherein said vector is a virus.

32. A method of inducing cell death comprising the administration of an agent capable of interfering with the binding of Kay-ligand to a receptor.

33. The method of claim 32 further comprising the administration of interferon-γ.

34. A method of treating, suppressing or altering an immune response involving a signaling pathway between Kay-ligand and its receptor, said method comprising the step of administering an effective amount of an agent capable of interfering with the association between Kay-ligand and its receptor.

35. The method of claim 34 wherein said immune response involves human adenocarcinoma cells.

36. A DNA sequence encoding Tumor-ligand or a fragment thereof.

37. A DNA sequence encoding Tumor-ligand, said sequence consisting essentially of SEQ. ID. NO. 5.

38. A DNA sequence consisting essentially of SEQ. ID. NO. 5 , said DNA encoding a polypeptide, said polypeptide consisting essentially of SEQ. ID. NO. 6.

39. A DNA sequence that hybridizes to at least a fragment of SEQ. ID NO. 5, said fragment comprising at least 20 consecutive bases, said DNA sequence encoding a polypeptide that is at least 30% homologous with an active site of Tumor-ligand.

40. A DNA sequence according to claim 2 wherein said sequence consists essentially of SEQ. ID. NO. 5 with conservative substitutions, alterations or deletions.

41. A recombinant DNA molecule comprising a DNA sequence encoding Tumor-ligand, said sequence operatively linked to an expression control sequence.

42. The molecule of claim 41 comprising SEQ. ID. NO. 5.

2843. A unicellular host transformed with a recombinant DNA molecule of claim 41 or 42.

44. A DNA sequence encoding Tumor-ligand having the amino acid sequence of SEQ. ID. NO. 6.

45. A method for producing substantially pure Tumor-ligand comprising the step of culturing the unicellular host of claim 43.

46. Tumor-ligand essentially free of normally associated animal proteins.

47. The Tumor-ligand of claim 46 consisting essentially of SEQ. ID. NO. 6.

48 A pharmaceutical composition comprising a therapeutically effective amount of Tumor-ligand or an active fragment thereof, and a pharmaceutically acceptable carrier.

49. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 48.

50. The pharmaceutical composition of claim 48 wherein said Tumor-ligand or active fragment thereof comprises SEQ. ID. NO. 6, or a biologically active fragment thereof.

51. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 50

29. A method for stimulating the immune system comprising administering the composition of claim 50

53. A method for suppressing the immune system comprising administering an effective amount of the pharmaceutical composition according to claim 50.

54. A method for treating cancer comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 50.

55. A method for identifying a receptor for the Tumor-ligand comprising:

a. providing a Tumor-ligand or a fragment thereof,

b. labeling said Tumor-ligand or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled Tumor-ligand of step b.

56. A soluble biologically active fragment of the Tumor-ligand of claim 55.

57. A polypeptide comprising an amino acid sequence that is encoded by a DNA selected from the group consisting of:

a. a DNA sequence comprising SEQ. ID. NO. 5;

b. a DNA sequence that hybridizes to the DNA defined in a. and coding on expression for a polypeptide that is at least 40% homologous with the Tumor-ligand of claim 12.

58 An antibody preparation that is reactive to Tumor-ligand or its receptor or biologically active fragments thereof.

59. The antibody preparation of claim 58 comprising monoclonal antibodies.

60. A method for producing an antibody preparation reactive to Tumor-ligand or its receptor comprising the step of immunizing an organism with Tumor-ligand or its receptor, or an antigenic fragment thereof.

61. An antisense nucleic acid against Tumor-ligand comprising a nucleic acid sequence hybridizing to at least a portion of SEQ. ID. NO. 5.

62. A pharmaceutical composition comprising an antibody preparation according to claim 58.

63. A method of expressing a gene in a mammalian cell comprising:

a. introducing a gene encoding Tumor-ligand into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

64. A method of treating a disorder related to Tumor-ligand in a mammal

a. introducing into a cell a therapeutically effective amount of a vector comprising a gene encoding the Tumor-ligand; and

b. expressing said gene in said mammalian cell.

65. The method of claim 64 wherein the mammal is a human.

66. The method of claim 64 wherein said vector is a virus.

67. A method of inducing cell death comprising the administration of an agent capable of interfering with the binding of Tumor-ligand to a receptor.

68. The method of claim 64 further comprising the administration of interferon-γ.

69. A method of treating, suppressing or altering an immune response involving a signaling pathway between Tumor-ligand and its receptor, said method comprising the step of administering an effective amount of an agent capable of interfering with the association between Tumor-ligand and its receptor.

70. The method of claim 69 wherein said immune response involves human adenocarcinoma cells.

Novel Ligands of the TNF Family

Abstract of the Disclosure

Kay-ligand, and Tumor-ligand, novel members of the tumor necrosis factor family (TNF), modified Kay-ligands and Tumor-ligands, and pharmaceutical compositions comprising them.

i. Smith et al. 1990; Kohno et al 1990; Loetscher et al 1990; Schall et al 1990.

ii. See Jones et al., 1989; Eck et al., 1992.

iii. K. Tracey, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 255 (1992)); A. Waage, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 275 (1992).

iv. G. D. Roodman, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 117 (1992).

v. A. Nakane, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 285 (1992); I. A. Clark et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 303 (1992); G. E. Grau et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 329 (1992); P-F. Piguet, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 341 (1992); G. H. Wong et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 371 (1992).

vi. S. Malik, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 407 (1992).

vii. D. A. Fox, Am. J. Med.,99, 82 (1995).

viii. D. Goeddel et al., Cold Spring Harbor Symposium Quant. Biol., 51, 597 (1986); G. Trinchieri, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 515 (1992).

ix. L. A. Tartaglia et al., Proc. Natl. Acad. Sci. USA, 88, 9292 (1991); L.A. Tartaglia and D. V. Goeddel, Immunol. Today, 13, 151 (1992).

x. B. Luettig et al., J. Immunol., 143, 4034 (1989); M. Kriegler et al., Cell, 53, 45 (1988).

xi. C. F. Ware et al., in Pathways for Cytolysis, G. M. Griffiths and J. Tschopp (Eds.), Springer-Verlag, Berlin, Heidelberg, p175-218 (1995).

xii. N. Paul et al., Ann. Rev. Immunol.,6,407 (1988).

xiii. P.D. Crowe et al., Science, 264, 707 (1994). (J. Browning et al., Cell, 72, 847 (1993); J. Browning et al., J. Immunol., 154, 33 (1995).

xiv. P. De Togni et al., Science, 264,703 (1993); T.A. Banks et al., J. Immunol., 155, 1685 (1995).

xv. J. Browning and A. Ribolini, J. Immunol.,143,1859 (1989): J. Browning et al., J. Exp. Med.,183, 867 (1996).

xvi. T. Suda et al., J. Immunol., 154, 3806 (1995)(T. Suda et al., J. Immunol., 154, 3806 (1995).

xvii. B.C. Trauth et al., Science, 245,301 (1989); S. Yonehara et al., J. Exp. Med., 169,1747 (1989); S. Nagata and P. Goldstein, Science, 267, 1449 (1995); M. H. Falk et al., Blood, 79, 3300 (1992).

xviii. F. Rieux-Laucat et al., Science, 268,1347 (1995); T. Takahashi et al., Cell, 76,969 (1994); R. Watanabe-Fukunaga et al., Nature, 356, 314 (1992).

xix. P. R. Galle and al., J. Exp. Med., 182, 1223 (1995).

xx. F. Silvestris and al., Clin. Immunol. Immunopathol., 75, 197 (1995).

xxi. P.D. Katsikis et al., J. Exp. Med.,181, 2029 (1995); A. D. Badley et al., J. Virol.,70, 199 (1996).

xxii. S. Wiley et al., Immunity, 3, 673 (1995).

xxiii. J. F. Gauchat et al., FEBS Lett., 315, 259 (1993); S. Funakoshi et al., Blood, 83, 2787 (1994).

xxiv. R. C. Allen et al., Science, 259, 990 (1993).

xxv. L. Biancone et al., Kidney-Int.,48, 458 (1995); C. Mohan et al., J. Immunol., 154, 1470 (1995).

xxvi. J. Ruby and al., Nature Medicine, 1, 437 (1995).

xxvii. Z. Wang et al., J. Immunol., 155, 3722 (1995); A. M. Cleary and al., J. Immunol., 155, 3329 (1995).

xxviii. S. Hess and H. Engelman, J. Exp. Med.,183, 159 (1996).

xxix. R. G. Goodwin et al, Cell, 73, 447 (1993); Goodwin et al, Eur. J. Immunol., 23, 2631 (1993); C. A. Smith et al., Cell, 73, 1349 (1993).

xxx. See for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477.

xxxi. See, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815.

33. M.T. Abreu-Martin, A. Vidrich, D.H. Lynch and S.R. Targan. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-" and ligation of Fas ligand. J. Immunol. 155: 4147-4154, 1995.

34. K. Agematsu, T. Kobata, F.-C. Yang, T. Nakazawa, K. Fukushima, M. Kitahara, T. Mori, K. Sugita, C. Morimoto and A. Komiyama. CD27/CD70 interaction directly drives B cell IgG and IgM synthesis. Eur. J. Immunol. 25: 2825-2829, 1995.

35. R. Amakawa, A. Hakem, T.M. Kundig, T. Matsuyama, J.J.L. Simard, E. Timms, A. Wakeham, H.-W. Mittruecker, H. Griesser, H. Takimoto, R. Schmits, A. Shahinian, P.S. Ohashi, J.M. Penninger and T.W. Mak. Impaired negative selection of T cells in Hodgkin=s disease antigen CD30-deficient mice. Cell 84: 551-562, 1996.

36. J.-L. Bodmer, K. Burens, P. Schneider, K. Hofmann, V. Steiner, M. Thome, T. Bornand, M. Hahne, M. Schroeter, K. Becker, A. Wilson, L.E. French, J.L. Browning, H.R. MacDonald, and J. Tschopp. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and fas (apo-1/CD95). Immunity 6: 79-88, 1997.

37. J. Brojatsch, J. Naughton, M.M. Rolls, K. Zingler and J.A.T. Young. Carl, a TNFR-related protein is a cellular receptor for cytopathic avian lleukosis-sarcoma viruses and mediates apoptosis. Cell 87: 845-855, 1996.

38. J.L. Browning, M.J. Androlewicz and C.F. Ware. Lymphotoxin and an associated 33-kDa glycoprotein are expresed on the surface of an activated human T cell hybridoma. J. Immunol. 147: 1230-7, 1991.

39. J.L. Browning, K. Miatkowski, D.A. Griffiths, P.R.Bourdon, C. Hession, C.M. Ambrose and W. Meier. Preparation and characterization of soluble recombinant heterotrimeric complexes of human lymphotoxins alpha and beta. J. Biol. Chem. 271: 8618-26, 1996.

40. J.E. Castro, J.A. Listman, B.A. Jacobson, Y. Wang, P.A. Lopez, S. Ju, P.W. Finn and D.L. Perkins. Fas Modulation of apoptosis during negative selection of thymocytes. Immunity 5: 617-627, 1996.

41. C.-Y.A. Chen and A.-B. Shyu. AU-rich elements: characterization and importance in mRNA degradation. Trends in Biol. Sci. 20: 465-470, 1995.

42. Y. Chicheportiche, C. Ody and P. Vassalli. Identification in mouse macrophages of a new 4 kb mRNA present in hematopoietic tissue which shares a short nucleotide sequence with erythropoietin mRNA. Biochim. Biophys. Res. Comm. 209: 1076-1081, 1995.

43. A.M. Chinnaiyan, K. O=Rourke, G.-L. Yu, R.H. Lyons, M. Garg, D.R. Duan, L. Xing, R. Gentz, J. Ni and V.M. Dixit. Signal transduction by DR3 a death-domain-containing receptor related to TNFR-1 and CD95. Science 274: 990-992, 1996.

44. P. DeTogni et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264: 703-7, 1994.

45. M.A. DeBenedette, N.R. Chu, K.E. Pollok, J. Hurtako, W.F. Wade, B.S. Kwon and T.H.Watts. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP. J. Exp. Med. 181: 985-992, 1995.

46. M. Degli-Esposti, T. Davis-Smith, W.S. Din, P.J. Smolak, R.G. Goodwin and C.A. Smith. Activation of the lymphotoxin-β receptor by cross-linking induces chemokine production and growth arrest in A375 melanoma cells. J. Immunol. 158: 1756-1762, 1997.

47. T.M. Foy, A. Aruffo, J. Bajorath, J.E. Buhlmann and R.J. Noelle. Immune regulation by CD40 and its ligand gp39. Ann. Rev. Immunol. 14: 591-617, 1996.

48. H.J. Gruss, N. Boiani, D.E. Williams, R.J. Armitage, C.A. Smith and R.G. Goodwin. Pleiotropic effects of the CD30 ligand on CD30-expressing cells and lymphoma cell lines. Blood 83: 2045-56, 1994.

49. H.J. Gruss and S.K. Dower. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood 85: 3378-404, 1995.

50. J. Kitson, T. Raven, Y.-P. Jiang, D.V. Goeddel, K.M. Giles, K.-T. Pun, C.J. Grinham, R.Brown and S.N. Farrow. A death domain-containing receptor that mediates apoptosis. Nature 384: 372-375, 1996.

51. S.Y. Lee, C.G. Park and Y. Choi. T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. J. Exp. Med. 183: 669-674, 1996.

52. R.I. Montgomery, M.S. Warner, B.J. Lum and P.G. Spear. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87: 427-436, 1996.

53. S. Nagata. Apoptosis by death factor. Cell 88: 355-365, 1997.

54. R.M. Pitti, S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore and A. Ashkenazi. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 1996.

55. C.A. Smith, T. Farrah and R.G. Goodwin. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76: 959-62, 1994.

56. G.L. Smith. Virus strategies for evasion of the host response to infection. Trends in Microbiol. 3: 81-88, 1994.

57. E.Strueber and W. Strober. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell independent humoral immune response. J. Exp. Med. 183: 979-989, 1996.

58. H.-K. Sytwu, R.S. Liblau and H.O. McDevitt. The roles of Fas/Apo-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor trangenic mice. Immunity 5: 17-30, 1996.

59. P. Vassalli. The pathophysiology of tumor necrosis factors. Ann. Rev. Immunol. 10: 411-452, 1992.

60. L. Zheng, G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch and M.J. Lenardo. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377: 348-351, 1995.

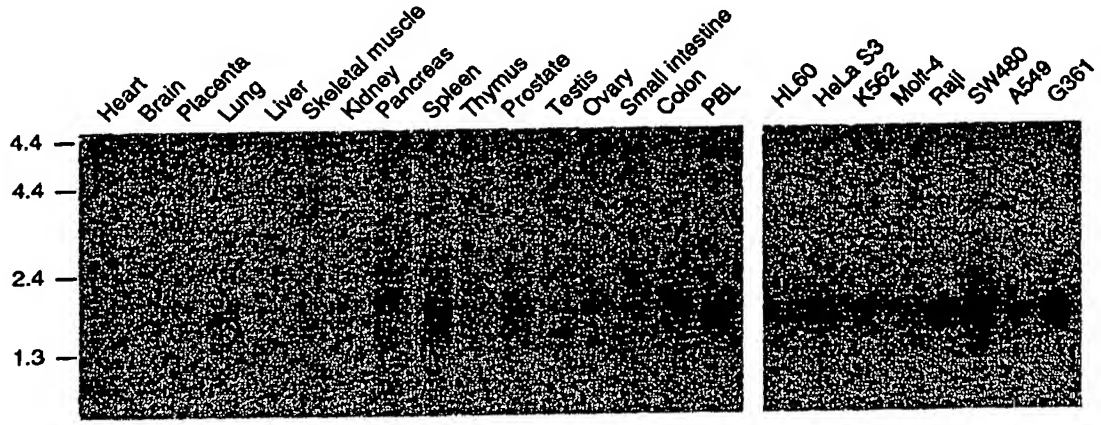
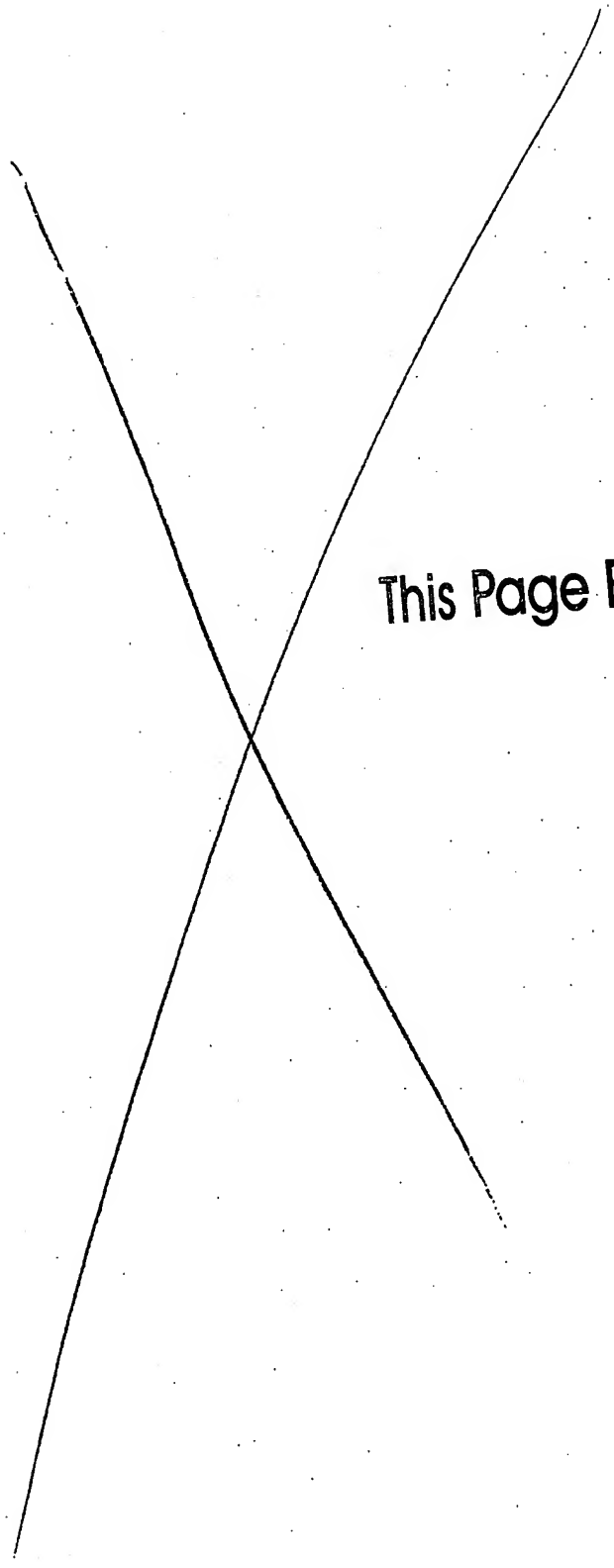


Fig. Northern Blot
Tumor-L



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PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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TITLE OF THE INVENTION (280 characters max)

APRIL-A NOVEL LIGAND IN THE TNF FAMILY

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification — *Number of Pages* 44

☒ Drawing(s) — *Number of Sheets* 5

☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE [signature] DATE 3/26/98

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APPLICATION

FOR

UNITED STATES PATENT

APPLICANTS: **Jurg Tschopp**

TITLE: **APRIL-A NOVEL LIGAND IN THE TNF FAMILY**

APRIL-NOVEL LIGANDS IN THE TNF FAMILY

BACKGROUND OF THE INVENTION

The present invention relates to novel ligand and polypeptides which are members of the Tumor Necrosis Factor Family. The novel ligand is designated April for "A Proliferation Inducing Ligand." These proteins or their receptors may have anti-cancer and/or immunoregulatory applications. Furthermore, cells transfected with the genes for these novel ligands may be used in gene therapy to treat tumors, autoimmune and inflammatory diseases or inherited genetic disorders, and blocking antitibodies to these proteins can have immunoregulatory applications.

BACKGROUND OF THE INVENTION

The tumor-necrosis factor (TNF)-related cytokines are mediators of host defense and immune regulation. Members of this family exist in membrane-anchored forms, acting locally through cell-to-cell contact, or as secreted proteins capable of diffusing to more distant targets. A parallel family of receptors signals the presence of these molecules leading to the initiation of cell death or cellular proliferation and differentiation in the target tissue. Presently, the TNF family of ligands and receptors has at least 13 recognized receptor-ligand pairs, including: TNF:TNF-R; LT-α:TNF-R; LT-α/β:LT-β-R; FasL:Fas; CD40L:CD40; CD30L:CD30; CD27L:CD27; OX40L:OX40 and 4-1BBL:4-1BB; trance/rankL: Light and Tweak. The DNA sequences encoding these ligands have only about 25% to about 30% identity in even the most related cases, although the amino acid relatedness is about 50%.

The defining feature of this family of cytokine receptors is found in the cysteine rich extracellular domain initially revealed by the molecular cloning of two distinct TNF receptors.[i] This family of genes encodes glycoproteins characteristic of Type I transmembrane proteins with an extracellular ligand binding domain, a single membrane spanning region and a cytoplasmic region involved in activating cellular functions. The cysteine-rich ligand binding region exhibits a tightly knit disulfide linked core domain, which, depending upon the particular family member, is repeated multiple times. Most receptors have four domains, although there may be as few as three, or as many as six.

Proteins in the TNF family of ligands are characterized by a short N-terminal stretch of normally short hydrophilic amino acids, often containing several lysine or arginine residues thought to serve as stop transfer sequences. Next follows a transmembrane region and an extracellular region of variable length, that separates the C-terminal receptor binding domain from the membrane. This region is sometimes referred to as the "stalk". The C-terminal binding region comprises the bulk of the protein, and often, but not always, contains glycosylation sites. These genes lack the classic signal sequences characteristic of type I membrane proteins, having type II membrane proteins with the C terminus lying outside the cell, and the short N-terminus residing in the cytoplasm. In some cases, e.g., TNF and LT-α, cleavage in the stalk region can occur early during protein processing and the ligand is then found primarily in secreted form. Most ligands, however, exist in a membrane form, mediating localized signaling.

The structure of these ligands has been well-defined by crystallographic analyses of TNF, LT-α, and CD40L. TNF and lymphotoxin-α (LT-α) are both structured into a sandwich of two anti-parallel β-pleated sheets with the "jelly roll" or Greek key topology.[ii] The rms deviation between the Cα and β-strand residues is 0.61 C, suggesting a high degree of similarity in their molecular topography. A structural feature emerging from molecular studies of CD40L, TNF and LT-α is the propensity to assemble into oligomeric complexes. Intrinsic to the oligomeric structure is the formation of the receptor binding site at the junction between the neighboring subunits creating a multivalent ligand. The quaternary structures of TNF, CD40L and LT-α have been shown to exist as trimers by analysis of their crystal structures. Many of the amino acids conserved between the different ligands are in stretches of the scaffold β-sheet. It is likely that the basic sandwich structure is preserved in all of these molecules, since portions of these scaffold sequences are conserved across the various family members. The quaternary structure may also be maintained since the subunit conformation is likely to remain similar.

TNF family members can best be described as master switches in the immune system controlling both cell survival and differentiation. Only TNF and LTα are currently recognized as secreted cytokines contrasting with the other predominantly membrane anchored members of the TNF family. While a membrane form of TNF has been well-characterized and is likely to have unique biological roles, secreted TNF functions as a general alarm signaling to cells more distant from the site of the triggering event. Thus TNF secretion can amplify an event leading to the well-described changes in the vasculature lining and the inflammatory state of cells. In contrast,

the membrane bound members of the family send signals though the TNF type receptors only to cells in direct contact. For example T cells provide CD40 mediated "help" only to those B cells brought into direct contact via cognate TCR interactions. Similar cell-cell contact limitations on the ability to induce cell death apply to the well-studied Fas system.

The ability to induce programmed cell death is an important and well-studied feature of several members of the TNF family. Fas mediated apoptosis appears to play a role in the regulation of autoreactive lymphocytes in the periphery and possibly the thymus (Castro et al., 1996) and recent work has also implicated the TNF and CD30 systems in the survival of T cells and large cell anaplastic lymphoma lines (Amakawa et al., 1996; Gruss et al., 1994; Sytwu et al., 1996; Zheng et al., 1995). We and others had previously shown the death of this line in response to TNF, Fas or LTß receptor signaling to have features of apoptosis (Abreu-Martin et al., 1995; Browning et al., 1996).

It appears that one can segregate the TNF ligands into three groups based on their ability to induce cell death (Table III). First, TNF, Fas ligand and TRAIL can efficiently induce cell death in many lines and their receptors mostly likely have good canonical death domains. Presumably the ligand to DR-3 (TRAMP/WSL-1) would also all into this category. Next there are those ligands which trigger a weaker death signal limited to few cell types and TWEAK, CD30 ligand and LTα1ß2 are examples of this class . How this group can trigger cell death in the absence of a canonical death domain is an interesting question and suggests that a separate weaker death signaling mechanism exists. Lastly, there are those members that cannot efficiently deliver a death signal. Probably all groups can have antiproliferative effects on some cell types consequent to inducing cell differentiation e.g. CD40 (Funakoshi et al., 1994)

The TNF family has grown dramatically in recent years to encompass at least 11 different signaling pathways involving regulation of the immune system. The expression patterns of TWEAK and TRAIL indicate that there is still more functional variety to be uncovered in this family. This aspect has been especially highlighted in recent the discovery of two receptors that affect the ability of rous sarcoma and herpes simplex virus to replicate as well as the historical observations that TNF has anti-viral activity and pox viruses encode for decoy TNF receptors (Brojatsch et al., 1996; Montgomery et al., 1996; Smith, 1994; Vassalli, 1992). The generation soluble TWEAK and the identification of the TWEAK receptor should provide the tools to elucidate the biological function of this interesting protein.

TNF is a mediator of septic shock and cachexia[iii], and is involved in the regulation of hematopoietic cell development.[iv] It appears to play a major role as a mediator of inflammation and defense against bacterial, viral and parasitic infections[v] as well as having antitumor activity.[vi] TNF is also involved in different autoimmune diseases.[vii] TNF may be produced by several types of cells, including macrophages, fibroblasts, T cells and natural killer cells.[viii] TNF binds to two different receptors, each acting through specific intracellular signaling molecules, thus resulting in different effects of TNF.[ix] TNF can exist either as a membrane bound form or as a soluble secreted cytokine.[x]

LT-α shares many activities with TNF, i.e. binding to the TNF receptors,[xi] but unlike TNF, appears to be secreted primarily by activated T cells and some β-lymphoblastoid tumors.[xii] The heteromeric complex of LT-α and LT-β is a membrane bound complex which binds to the LT-β receptor.[xiii] The LT system (LTs and LT-R) appears to be involved in the development of peripheral lymphoid organs since genetic disruption of LT-β leads to disorganization of T and B cells in the spleen and an absence of lymph nodes.[xiv] The LT-β system is also involved in cell death of some adenocarcinoma cell lines.[xv]

Fas-L, another member of the TNF family, is expressed predominantly on activated T cells.[xvi] It induces the death of cells bearing its receptor, including tumor cells and HIV-infected cells, by a mechanism known as programmed cell death or apoptosis.[xvii] Furthermore, deficiencies in either Fas or Fas-L may lead to lymphoproliferative disorders, confirming the role of the Fas system in the regulation of immune responses.[xviii] The Fas system is also involved in liver damage resulting from hepatitis chronic infection[xix] and in autoimmunity in HIV-infected patients.[xx] The Fas system is also involved in T-cell destruction in HIV patients.[xxi] TRAIL, another member of this family, also seems to be involved in the death of a wide variety of transformed cell lines of diverse origin.[xxii]

CD40-L, another member of the TNF family, is expressed on T cells and induces the regulation of CD40-bearing B cells.[xxiii] Furthermore, alterations in the CD40-L gene result in a disease known as X-linked hyper-IgM syndrome.[xxiv] The CD40 system is also involved in different autoimmune diseases[xxv] and CD40-L is known to have antiviral properties.[xxvi] Although the CD40 system is involved in the rescue of apoptotic B cells,[xxvii] in non-immune cells it induces apoptosis[xxviii]. Many additional lymphocyte members of the TNF family are also involved in costimulation.[xxix]

Generally, the members of the TNF family have fundamental regulatory roles in controlling the immune system and activating acute host defense systems. Given the current progress in manipulating members of the TNF family for therapeutic benefit, it is likely that members of this family may provide unique means to control disease. Some of the ligands of this family can directly induce the apoptotic death of many transformed cells e.g. LT, TNF, Fas ligand and TRAIL (Nagata, 1997). Fas and possibly TNF and CD30 receptor activation can induce cell death in nontransformed lymphocytes which may play an immunoregulatory function (Amakawa et al., 1996; Nagata, 1997; Sytwu et al., 1996; Zheng et al., 1995). In general, death is triggered following the aggregation of death domains which reside on the cytoplasmic side of the TNF receptors. The death domain orchestrates the assembly of various signal transduction components which result in the activation of the caspase cascade (Nagata, 1997). Some receptors lack canonical death domains, e.g. LTb receptor and CD30 (Browning et al., 1996; Lee et al., 1996) yet can induce cell death, albeit more weakly. It is likely that these receptors function primarily to induce cell differentiation and the death is an aberrant consequence in some transformed cell lines, although this picture is unclear as studies on the CD30 null mouse suggest a death role in negative selection in the thymus (Amakawa et al., 1996). Conversely, signaling through other pathways such as CD40 is required to maintain cell survival. Thus, there is a need to identify and characterize additional molecules which are members of the TNF family thereby providing additional means of controlling disease and manipulating the immune system.

It has been suggested that certain members of the TNF family may provide therapeutic anti-tumor benefits, for example, in combination with IL-2. (See, e.g. U.S. 5,425,940). However, to date, no completely satisfactory treatment for cancer is known. Combination chemotherapy is commonly used in the clinic and in research, for example with antimetabolites, alkylating agents, antibiotics, general poisons, etc. Such drugs are administered alone or in combination in an attempt to obtain a cytotoxic effect on cancers, and/or to reduce or eliminate the emergence of drug-resistant cells, and to reduce side effects.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to a novel polypeptide referred to as APRIL, which substantially obviates one or more of the problems due to the limitations and disadvantages of the related art. The inventors have discovered a new member of the TNF

family of cytokines, and defined both the human amino acid sequence of the protein, as well as the DNA sequences encoding these proteins. The claimed invention may be used to identify new diagnostics and therapeutics for numerous diseases and conditions as discussed in more detail below, as well as to obtain information about, and manipulate, the immune system and its processes. Additionally, the invention may be involved in the induction of cell death in carcinomas.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent form the description, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof, as well as in the appended drawings.

Thus, to achieve these and other advantages, and in accordance with the purpose of the invention, as embodied and broadly described herein, the invention includes DNA sequences encoding APRIL. Specifically, the invention relates to DNA sequences which encode APRIL, (SEQ. ID. NO. 1). Additionally, the claimed invention relates to the amino acid sequences of this novel ligand. The amino acid sequence of APRIL is set forth in SEQ. ID. NO.: 1. In other embodiments, the invention relates to sequences that have at least 50% homology with DNA sequences encoding the C terminal receptor binding domain of this ligand and when hybridize to the claimed DNA sequences or fragments thereof, and which encode APRIL having the sequence identified in SEQ. ID. NO. 2, or a protein having similar biological activity.

The invention in certain embodiments furthermore relates to DNA sequences encoding APRIL where the sequences are operatively linked to an expression control sequence. Any suitable expression control sequences are useful in the claimed invention, and can easily be selected by one skilled in the art.

The invention also contemplates recombinant DNAs comprising a sequence encoding APRIL or fragments thereof, as well as hosts with stably integrated APRIL sequences introduced into their genome, or possessing episomal elements. Any suitable host may be used in the invention, and can easily be selected by one skilled in the art without undue experimentation.

In other embodiments, the invention relates to methods of producing substantially pure APRILs comprising the step of culturing transformed hosts. In yet other embodiments, the invention relates to APRIL essentially free of normally associated animal proteins.

The invention encompasses APRIL ligands having the amino acid sequence identified in SEQ. ID. NO. 2, as well as fragments or homologs thereof. In various embodiments, the amino acid and/or the DNA sequences may comprise conservative insertions, deletions and substitutions, as further defined below or may comprise fragments of said sequences.

The invention relates in other embodiments to soluble constructs comprising APRIL which may be used to directly trigger APRIL mediated pharmacological events. Such events may have useful therapeutic benefits in the treatment of cancer, tumors or the manipulation of the immune system to treat immunologic diseases. Soluble forms of the claimed ligands could be genetically reengineered to incorporate an easily recognizable tag, thereby facilitating the identification of the receptors for these ligands.

In yet other embodiments the invention relates to methods of gene therapy using the genes for APRIL as disclosed and claimed herein.

The pharmaceutical preparations of the invention may, optionally, include pharmaceutically acceptable carriers, adjuvants, fillers, or other pharmaceutical compositions, and may be administered in any of the numerous forms or routes known in the art.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory, and are intended to provide further explanation of the invention as claimed.

The accompanying drawings are included to provide a further understanding of the invention, and are incorporated in, and constitute a part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure legends

Figure 1. (A) Predicted amino acid sequence of human APRIL. The predicted transmembrane region (TM, boxed), the potential N-linked glycosylation site (star) and the N-terminus of the recombinant soluble APRIL (sAPRIL) are indicated. (B) Comparison of the extracellular protein sequence of APRIL and some members of the TNF ligand family. Identical and homologuous

residues are represented in black and shaded boxes, respectively. TNFa, tumor necrosis factor α, LTa, lymphotoxin α, FasL, Fas (CD95) ligand, TRANCE, RANK ligand.

Figure 2. Expression of APRIL (A) Northern blots (2 μg poly A^+ RNA per lane) of various human tissues were probed with APRIL cDNA. (B) APRIL mRNA expression in various tumor cell lines: promyelocitic leukemia HL 60 ; HeLa Cell S3; chronic myelogenous leukemia K562; lymphoblastic leukemia Molt-4; Burkitt's lymphoma Raji; colorectal adenocarcinoma A459; melanoma G361. (C) APRIL mRNA expression in four different human tumors (T) and normal tissues (N). The 18S rRNA band shows equal loading. (D) APRIL mRNA expression in primary colon carcinoma. In situ hybridization revealed abundant APRIL message in human colon carcinoma as compared to normal colon tissue. Colon tumor tissue sections and adjacent normal colon tissue were hybridized to antisense APRIL ^{35}S-labeled cRNA, and as control, colon tumor tissue sections were also hybridized to sense APRIL ^{35}S cRNA (negative control). The upper panels are dark field micrographs, the lower panels are the corresponding light field micrographs.

Figure 3. APRIL stimulates cell growth. (A) Dose dependent increase of proliferation of Jurkat (human leukemia T cells), as determined 24 hrs after addition of soluble APRIL. Controls are Fas ligand (FasL), TWEAK and no ligand (Control) (left panel, cell viability; right panel, ^3H-Thymidine incorperation). (B) Influence of immunodepletion of FLAG-tagged APRIL on tumor cell growth. The proliferative effect of FLAG-tagged APRIL is neutralized by anti-FLAG antibodies, but not by anti-myc antibodies. (C) Effect of APRIL on the proliferation rate of Raji (human Burkitt lymphoma B cells), A20 cells (mouse B lymphoma), BJAB (human B lymphoma), COS (canine epithelial cells), MCF-7 (human breast adenocarcinoma), HeLa (human epitheloid carcinoma) and ME260 (human melanoma). (D) Influence of fetal calf serum concentration on APRIL-induced proliferation of Jurkat cells.

Figure 4. APRIL accelerates tumor growth. (A) Characterization of APRIL-transfected NIH-3T3 clones. FLAG-APRIL levels of the various clones were analyzed by Western blotting using an anti-FLAG antibody. The arrow points to the APRIL protein, the high molecular weight

protein is detected non-specifically (B) APRIL-expressing NIH-3T3 clones grow faster than mock-transfeced clones. (C) Increased tumor growth of APRIL-expressing NIH-3T3 clones. NIH-3T3 cells (1 x 10^5 cells) and APRIL (NIH-AP, 2 different clones) transfectants (1 x 10^6 cells) were injected subcutaneously into nude mice, and tumor growth monitored.

DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention. This invention relates to DNA sequences that code for human or mouse APRIL, fragments and homologs thereof, and expression of those DNA sequences in hosts transformed with them. The invention relates to uses of these DNA sequences and the peptides encoded by them. Additionally, the invention encompasses both human and mouse amino acid sequences for and APRIL, or fragments thereof, as well as pharmaceutical compositions comprising or derived from them. The invention relates to methods of stimulating cell growth with APRIL, or, alternatively, methods of inhibiting tumorogenesis using antibodies directed against APRIL or a receptor of APRIL.

A. DEFINITIONS

"Homologous", as used herein, refers to the sequence similarity between sequences of molecules being compared. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

As used herein, the term "cancer" refers to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer, mammary adenocarcinoma, pharyngeal squamous cell

carcinoma, and gastrointestinal or stomach cancer. Preferably, the cancer is leukemia, mastocytoma, melanoma, lymphoma, mammary adenocarcinoma, and pharyngeal squamous cell carcinoma

A "purified preparation" or a "substantially pure preparation" of a polypeptide, as used herein, means a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from other substances, e.g., antibodies, matrices, etc., which are used to purify it.

"Transformed host" as used herein is meant to encompass any host with stably integrated sequence, i.e. a sequence encoding APRIL, introduced into its genome.

A "treatment", as used herein, includes any therapeutic treatment, e.g., the administration of a therapeutic agent or substance, e.g., a drug.

A "substantially pure nucleic acid", e.g., a substantially pure DNA, is a nucleic acid which is one or both of: (1) not immediately contiguous with either one or both of the sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or (2) which is substantially free of a nucleic acid sequence with which it occurs in the organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding APRIL.

The terms "peptides", "proteins", and "polypeptides" are used interchangeably herein.

"Biologically active" as used herein, means having an in vivo or in vitro activity which may be performed directly or indirectly. Biologically active fragments of APRIL may have, for example, 70% amino acid homology with the active site of APRIL, more preferably at least 80%, and most preferably, at least 90% homology. Identity or homology with respect to APRIL is defined herein as the percentage of amino acid residues in the candidate sequence which are identical to the APRIL residues in SEQ. ID. NOS. 1 or 2.

"Ligand" as used herein generically refers to APRIL. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell

culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature.

Introduction:

APRIL, a novel member of the TNF family, is described in detail herein. The inventors have found that while transcript of APRIL are of low abundance in normal tissues, high levels of mRNA are detected in several tumor cell lines, as well as in colon carcinomas, metastatic lymphomas and thyroid tumors. In vitro, the addition of recombinant APRIL stimulates the proliferation of various cell lines. Moreover, transfection of APRIL into NIH-3T3 cells dramatically accelerated tumor growth in nude mice when compared to mock transfectants. The expression and growth stimulating effect of APRIL on tumor cells in vitro and in vivo suggests that APRIL is implicated in tumorigenesis.

APRIL appears to be unique among the members of the TNF family as it is both abundantly expressed in tumor cells and stimulates growth of many different tumor cell lines given the apparent role of APRIL is tumorigenesis, the antagonistic antibodies to APRIL, or the APRIL receptor, will provide novel approaches to cancer treatment.

B. DNA SEQUENCES OF THE INVENTION

As described herein, one aspect of the invention features a substantially pure (or recombinant) nucleic acid which includes a nucleotide sequence encoding APRIL, such as the DNA described in SEQ. ID. NO. 1 and/or equivalents of such nucleic acids. The term nucleic acid as used herein can include fragments and equivalents, such as, for example, sequences encoding functionally equivalent peptides. Equivalent nucleotide sequences may include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, mutations, etc. and include sequences that differ from the nucleotide sequence encoding APRIL shown in SEQ. ID NO: 1 due to the degeneracy of the genetic code.

The invention will be described generally by reference to the human sequences, although one skilled in the art will understand that the mouse sequences or sequences encoding APRIL from other species having a high level of homology with human, and are encompassed herein. The human proteins appear to have all of the characteristics of the TNF family, i.e., a type II

membrane protein organization and conservation of the sequence motifs involved in the folding of the protein into the TNF anti-parallel β-sheet structure.

The sequences of the invention can be used to prepare a series of DNA probes that are useful in screening various collections of natural and synthetic DNAs for the presence of DNA sequences that code for APRIL, or fragments or derivatives thereof. One skilled in the art will recognize that reference to APRIL as used herein, refers also to biologically active derivatives, fragments or homologs thereof.

The DNA sequences of the invention coding on APRIL can be employed to produce the claimed peptides on expression in various prokaryotic and eukaryotic hosts transformed with them. These peptides may be used in anti-cancer, and immunoregulatory applications. In general, this comprises the steps of culturing a host transformed with a DNA molecule containing the sequence encoding APRIL, operatively-linked to an expression control sequence.

The DNA sequences and recombinant DNA molecules of the present invention can be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal or synthetic DNA sequences. The expression vectors of the invention are characterized by at least one expression control sequence that may be operatively linked to the APRIL DNA sequence inserted in the vector, in order to control and to regulate the expression of the DNA sequence.

Furthermore, within each expression vector, various sites may be selected for insertion of a sequence of the invention. The sites are usually designated by a restriction endonuclease which cuts them, and these sites and endonucleases are well recognized by those skilled in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the desired DNA fragment. Instead, the vector may be cloned to the fragment by alternate means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment, and its operative linking therein to an expression control sequence, is determined by a variety of factors. These factors include, but are not limited to, the size of the protein to be expressed, the susceptibility of the desired protein to proteolytic degradation by host cell enzymes, number of sites susceptible to a particular restriction enzyme, contamination or binding of the protein to be expressed by host cell proteins which may prove difficult to remove during purification. Additional factors which may be considered include expression characteristics such as the location of start and stop codons

relative to the vector sequences, and other factors which will be recognized by those skilled in the art. The choice of a vector and insertion site for the claimed DNA sequences is determined by a balancing of these factors, not all selections being equally effective for a desired application. However, it is routine for one skilled in the art to analyze these parameters and choose an appropriate system depending on the particular application.

One skilled in the art can readily make appropriate modifications to the expression control sequences to obtain higher levels of protein expression, i.e. by substitution of codons, or selecting codons for particular amino acids that are preferentially used by particular organisms, to minimize proteolysis or to alter glycosylation composition. Likewise, cysteines may be changed to other amino acids to simplify production, refolding or stability problems.

Thus, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention. However, a particular selection of a host/expression vector combination may be made by those of skill in the art. Factors one may consider include, for example, the compatibility of the host and vector, toxicity to the host of the proteins encoded by the DNA sequence, ease of recovery of the desired protein, expression characteristics of the DNA sequences and expression control sequences operatively linked to them, biosafety, costs and the folding, form or other necessary post-expression modifications of the desired protein.

APRIL produced by hosts transformed with the sequences of the invention, as well as native APRIL purified by the processes of this invention, or produced from the claimed amino acid sequences, are useful in a variety of compositions and methods for anticancer, antitumor and immunoregulatory applications. They are also useful in therapy and methods directed to other diseases.

This invention also relates to the use of the DNA sequences disclosed herein to express APRIL under abnormal conditions, i.e. in a gene therapy setting. Additionally, APRIL may be expressed in tumor cells under the direction of promoters appropriate for such applications. Such expression could enhance anti-tumor immune responses or directly affect the survival of the tumor. APRIL is also likely to affect the survival of an organ graft by altering the local immune response. In this case, the graft itself or the surrounding cells would be modified with an engineered gene encoding APRIL.

Another aspect of the invention relates to the use of the isolated nucleic acid encoding either APRIL in "antigens" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular conditions with the cellular mRNA and/or DNA encoding the APRIL sequence of interest, so as to inhibit expression of the encoded protein, i.e. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to a range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA which is complementary to at least a portion of the cellular mRNA which encodes APRIL. Alternatively, the antisense construct can be an oligonucleotide probe which is generated ex vivo. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, and are therefor stable in vivo. Exemplary nucleic acids molecules for use as antisense oligonucleotides are phosphoramidates, phosphothioate and methylphosphonate analogs of DNA (See, e.g., 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van Der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48: 2659-2668, specifically incorporated herein by reference.

C. APRIL AND AMINO ACID SEQUENCES THEREFOR

APRIL, as discussed above, is a member of the TNF family. The protein, fragments or homologs of APRIL may have wide therapeutic and diagnostic applications as discussed in more detail below.

Although the precise three dimensional structure of APRIL is not known, it is predicted that, as a member of the TNF family, it may share certain structural characteristics with other members of the family.

Comparison of the claimed APRIL sequence with other members of the human TNF family reveals considerable structural similarity. All the proteins share several regions of

sequence conservation in the extracellular domain. The overall sequence homology of the extracellular domain of APRIL show the highest homology to FasL (21% amino acid identities), TNFα (20%), LT-β (18%), followed by TRAIL, TWEAK and TRANCE (15%). Figure 2.

The novel polypeptides of the invention specifically interact with a receptor, which has not yet been identified. However, the peptides and methods disclosed herein enable the identification of receptors which specifically interact with APRIL or fragments thereof.

The claimed invention in certain embodiments includes peptides derived from APRIL which have the ability to bind to its receptors. Fragments of APRIL can be produced in several ways, e.g., recombinantly, by PCR, proteolytic digestion or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end or both ends of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNAs which encode a variety of fragments. DNAs which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above discussed methods.

Polypeptide fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f- moc or t-boc chemistry. For example, peptides and DNA sequences of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragment, or divided into overlapping fragments of a desired length. Methods such as these are described in more detail below.

D. Generation of Soluble Forms of APRIL

Soluble forms of APRIL can often signal effectively and hence can be administered as a drug which now mimics the natural membrane form. It is possible that APRIL as claimed herein is naturally secreted as a soluble cytokines, however, if not, one can reengineer the gene to force secretion. To create a soluble secreted form of APRIL, one would remove at the DNA level the N-terminus transmembrane regions, and some portion of the stalk region, and replace them with a type I leader or alternatively a type II leader sequence that will allow efficient proteolytic cleavage in the chosen expression system. A skilled artisan could vary the amount of the stalk region retained in the secretion expression construct to optimize both receptor binding properties and secretion efficiency. For example, the constructs containing all possible stalk lengths, i.e. N-

terminal truncations, could be prepared such that proteins starting at amino acids 81 to 139 would result. The optimal length stalk sequence would result from this type of analysis.

E. Generation of Antibodies Reactive with APRIL

The invention also includes antibodies specifically reactive with APRIL or its receptor. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers, or other techniques are well known in the art.

An immunogenic portion of APRIL or its receptor can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of APRIL, or its receptor, e.g. antigenic determinants of a polypeptide of SEQ. ID. NO.: 2, or a closely related human or non-human mammalian homolog (e.g. 70, 80 or 90 percent homologous, more preferably at least 95 percent homologous). In yet a further preferred embodiment of the present invention, the anti-APRIL or anti-APRIL-receptor antibodies do not substantially cross react (i.e. react specifically) with a protein which is e.g., less than 80 percent homologous to SEQ. ID. NO. 2; preferably less than 90 percent homologous with SEQ. ID. NO.: 2; and, most preferably less than 95 percent homologous with SEQ. ID. NO.: 2. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is less than 10 percent, more preferably less than 5 percent, and even more preferably less than 1 percent, of the binding affinity for a protein of SEQ. ID. NO. 2.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with APRIL, or its receptor. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibodies of the present invention are further intended to include biospecific and chimeric molecules having anti-APRIL or anti-APRIL -receptor activity. Thus,

both monoclonal and polyclonal antibodies (Ab) directed against APRIL and its receptor, and antibody fragments such as Fab` and $F(ab`)_2$, can be used to block the action of APRIL and its respective receptor.

Various forms of antibodies can also be made using standard recombinant DNA techniques. (Winter and Milstein, Nature 349: 293-299 (1991) specifically incorporated by reference herein.) For example, chimeric antibodies can be constructed in which the antigen binding domain from an animal antibody is linked to a human constant domain (e.g. Cabilly et al., U.S. 4,816,567, incorporated herein by reference). Chimeric antibodies may reduce the observed immunogenic responses elicited by animal antibodies when used in human clinical treatments.

In addition, recombinant "humanized antibodies" which recognize APRIL, or its receptor can be synthesized. Humanized antibodies are chimeras comprising mostly human IgG sequences into which the regions responsible for specific antigen-binding have been inserted. Animals are immunized with the desired antigen, the corresponding antibodies are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized antibodies minimize the use of heterologous (i.e. inter species) sequences in human antibodies, and thus are less likely to elicit immune responses in the treated subject.

Construction of different classes of recombinant antibodies can also be accomplished by making chimeric or humanized antibodies comprising variable domains and human constant domains (CH1, CH2, CH3) isolated from different classes of immunoglobulins. For example, antibodies with increased antigen binding site valencies can be recombinantly produced by cloning the antigen binding site into vectors carrying the human chain constant regions. (Arulanandam et al., J. Exp. Med., 177: 1439-1450 (1993), incorporated herein by reference.)

In addition, standard recombinant DNA techniques can be used to alter the binding affinities of recombinant antibodies with their antigens by altering amino acid residues in the vicinity of the antigen binding sites. The antigen binding affinity of a humanized antibody can be increased by mutagenesis based on molecular modeling. (Queen et al., Proc. Natl. Acad. Sci. 86: 10029-33 (1989) incorporated herein by reference.

F. Generation of Analogs: Production of Altered DNA and Peptide Sequences

Analogs of APRIL can differ from the naturally occurring Ligands in amino acid sequence, or in ways that do not involve sequence, or both. Non-sequence modifications include in vivo or in vitro chemical derivatization of APRIL. Non-sequence modifications include, but are not limited to, changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

Preferred analogs include, APRIL or biologically active fragments thereof, whose sequences differ from the sequence given in SEQ. ID NO. 2, by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity of APRIL. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g. substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and, phenylalanine, tyrosine.

TABLE 1

CONSERVATIVE AMINO ACID REPLACEMENTS

for amino Acid	code	replace with any of:
Alanine	A	D-Ala, Gly, Beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met

Lysine	K	D-Lys, Arg, D-Arg, Homo-arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3, 4 or 5-phenylproline, cis-3, 4, or 5-phenylproline
Proline	P	D-Pro, L-I-thoazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Useful methods for mutagenesis include PCR mutagenesis and saturation mutagenesis as discussed in more detail below. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences.

-PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity can be used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized can be amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments can be inserted into appropriate cloning vectors to provide random mutant libraries.

-Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as of a protein can be prepared by random mutagenesis of DNA which those that alter function, can be obtained. The distribution of point mutations is not biased toward conserved sequence elements.

-Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art[xxx] Such techniques have been employed in the directed evolution of other proteins[xxxi].

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by

(1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

-Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989) specifically incorporated by reference. In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions can then be refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

-Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983) incorporated herein by reference. Briefly, the desired DNA can be altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily

synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]) incorporated herein by reference.

-Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]) incorporated herein by reference. The starting material can be a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

-Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors,

and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, binding to APRIL or its receptor, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

The invention also provides for reduction of the protein binding domains of the claimed polypeptides or their receptors, to generate mimetics, e.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of APRIL with its respective receptor. The critical residues of the APRIL involved in molecular recognition of a receptor polypeptide or of a downstream intracellular protein, can be determined and used to generate APRIL or its receptor-derived peptidomimetics which competitively or noncompetitively inhibit binding of APRIL with a receptor. (see, for example, "Peptide inhibitors of human papilloma virus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A), specifically incorporated herein by reference.

By making available purified and recombinant APRIL, the present invention provides assays which can be used to screen for drug candidates which are either agonists or antagonists of the normal cellular function, in this case, of APRIL or its receptor. In one embodiment, the assay evaluates the ability of a compound to modulate binding between APRIL and a receptor. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by the skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

Isolation of a receptor binding to APRIL

Ligands of the TNF family can be used to identify and clone receptors. With the described APRIL sequences, one could fuse the 5' end of the extracellular domain which constitutes the receptor binding sequence to a marker or tagging sequence and then add a leader sequence that will force secretion of APRIL in any of a number of expression systems. One example of this technology is described by Browning et al., (1996) (JBC 271, 8618-8626) where the LT-β ligand was secreted in such a form. The VCAM leader sequence was coupled to a short myc peptide tag followed by the extracellular domain of the LT-β. The VCAM sequence is used to force secretion of the normally membrane bound LT-β molecule. The secreted protein retains a myc tag on the N-terminus which does not impair the ability to bind to a receptor. Such a secreted protein can be expressed in either transiently transfected Cos cells or a similar system, e.g., EBNA derived vectors, insect cell/baculovirus, picchia etc. The unpurified cell supernatant can be used as a source of the tagged ligand.

Cells expressing the receptor can be identified by exposing them to the tagged ligand. Cells with bound ligand are identified in a FACS experiment by labeling the myc tag with an anti-myc peptide antibody (9E10) followed by phycoerythrin (or a similar label) labeled anti-mouse immunoglobulin. FACS positive cells can be readily identified and would serve as a source of RNA encoding for the receptor. An expression library would then be prepared from this RNA via standard techniques and separated into pools. Pools of clones would be transfected into a suitable host cell and binding of the tagged ligand to receptor positive transfected cells determined via microscopic examination, following labeling of bound myc peptide tag with an enzyme labeled anti-mouse Ig reagent, i.e. galactosidase, alkaline phosphatase or luciferase labeled antibody. Once a positive pool has been identified, the pool size would be reduced until the receptor encoding cDNA is identified. This procedure could be carried out with either the mouse or human APRIL, as one may more readily lead to a receptor.

G. METHODS OF TREATMENT AND PHARMACEUTICAL COMPOSITIONS

The methods of the invention for the treatment of cancers involve the adminstration to a patient, preferably a mammalian host, such as a dog, cat, or human, an effective amount of a claimed composition comprising a blocking agent capable of interfering with the asociation

between APRIL and its receptor. Such blocking agents include, but are not limited to soluble APRIL anti-APRIL antibodies, anti-APRIL receptor antibodies, or biologically active fragments thereof. Blocking agents may preferably comprise a receptor IG fusion protein, which can be constructed by methods known to those of skill in the art.

The methods of the invention are useful for treating all cancers, including, but not limited to, cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancerm mammary adenocarcinoma, pharyngeal squamous cell carcinoma, and gastrointestinal or stomach cancer.

Pharmaceutical compositions of the invention may comprise a therapeutically effective amount of APRIL, or its receptor, or fragments or mimetics thereof, and, optionally may include pharmaceutically acceptable carriers. Accordingly, this invention provides methods for treatment of cancer, and methods of stimulating, or in certain instances, inhibiting the immune system, or parts thereof by administering a pharmaceutically effective amount of a compound of the invention or its pharmaceutically acceptable salts or derivatives. It should of course by understood that the compositions and methods of this invention can be used in combination with other therapies for various treatments.

The compositions can be formulated for a variety of routes of administration, including systemic, topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the compositions of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compositions may be formulated in solid form and, optionally, redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

The compositions can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration, bile salts, fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the compositions are formulated into conventional oral administration forms such

as capsules, tablets, and tonics. For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams as known in the art.

The dose and dosing regimen will depend on the type of cancer, the patient and the patient's history. The amount must be effective to treat, suppress, or alter the progression of cancer. The doses may be single doses or multiple doses. If multiple doses are employed, as preferred, the freuence of administration will depend, for rexample, on the type of host and and type of cancer, dosage amounts etc. For some types of cancers or cancer lines, dily administration wil be effective, whereas for others, administration every other day or every third day will be effective. The amount of active compound administered at one time or over the course of treatment will depend on many factors. For example, the age and size of the subject, the severity and course of the disease being treated, the manner and form of administration, and the judgments of the treating physician. However, an effective dose may be in the range of from about 0.005 to about 5 mg/kg/day, preferably about 0.05 to about 0.5 mg/kg/day. The dosage amount which will be most effective will be one which results in no tumor appearance or complete regression of the tumor, and is not toxic to the patient. One skilled in the art will recognize that lower and higher doses may also be useful.

Gene constructs according to the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of APRIL.

Expression constructs of the APRIL can be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the gene for APRIL to cells in vivo. Approaches include insertion of the gene in viral vectors which can transfect cells directly, or delivering plasmid DNA with the help of, for example, liposomes, or intracellular carriers, as well as direct injection of the gene construct. Viral vector transfer methods are preferred.

A pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA, RNA or amino acid sequences to

which they specifically bind. In other aspects, the claimed invention may be used to evaluate a chemical entity for its ability to interact with, e.g., bind or physically associate with APRIL or a fragment thereof. The method includes contacting the chemical entity with APRIL, and evaluating the ability of the entity to interact with APRIL. Additionally, APRIL can be used in methods of evaluating naturally occurring APRIL or receptors of APRIL, as well as to evaluate chemical entities which associate or bind with receptors of APRIL.

In certain aspects, the claimed invention features a method for evaluating a chemical entity for the ability to modulate the interaction between APRIL and its respective receptor. The method includes combining a receptor for APRIL, and APRIL under conditions wherein the pair is capable of interacting, adding the chemical entity to be evaluated and detecting the formation or dissolution of complexes. These modulating agents may be further evaluated in vitro, e.g. by testing its activity in a cell free system, and then, optionally administering the compound to a cell or animal, and evaluating the effect.

I. EXAMPLES

Example 1

Northern blot analysis of APRIL revealed that the expression of APRIL was weak and restricted only to a few tissues (Fig. 2A). Two transcripts of 2.1 kb and 2.4 kb were found in the prostate, whereas PBLs revealed a shorter transcript of 1.8 kb. Northern blot analysis was performed by using Human Multiple Tissue Northern Blots I and II (Clontech #7760-1 and #7759-1), Human Cancer Cell Line MTN Blot (Clontech #7757-1) and Human Tumor Panel Blot V (Invitrogen D3500-01). The membranes were incubated in ExpressHyb hybridization solution (Clontech #8015-1) for at least 1 hour at 62°C. The random-primed cDNA probe (Boehringer Mannheim) was synthesized using cDNA corresponding to the extracellular domain of APRIL as template. The heat-denatured cDNA probe was added at 1.5 $x10^6$ cpm/ml in fresh ExpressHyb. The membrane was hybridized 12-24 hr at 62°C, washed three times in 2xSSC containing 0.05% SDS and exposed at -70°C. Northern blot analysis of APRIL revealed that the

expression of APRIL was weak and restricted only to a few tissues. Two transcripts of 2.1 kb and 2.4 kb were found in th eprostrate, whereas PBLs revealed a shorter transcript of 1.8 kb.

A longer exposure time revealed the 2.1 kb APRIL mRNA in colon, spleen, and pancreas (data not shown). This restricted distribution of the APRIL mRNA is consistent with the origin of cDNA clones currently available in the EST database. Of the 23 clones identified only two were derived from normal tissues (pregnant uterus, pancreatic islands). Remarkably, the remainder of the EST-clones (21 clones, 91%) were present in cDNA libraries generated from tumors or tumor-derived cell lines (Ovary tumor, 11; prostate tumor, 3; Gessler Wilms tumor, 1; colon carcinoma, 1; endometrial tumor, 1; parathyroid tumors, 1; pancreas tumor, 1; T-cell lymphoma, 1; LNCAP adenocarcinoma derived cell line, 1). This prompted us to test transformed cell lines for the expression of APRIL mRNA (Figure 2B), and indeed, all cell lines strongly expressed the 2.1 kb transcript of APRIL.

Highest APRIL-specific signals were detected in the colorectal adenocarcinoma SW480, the Burkitt's lymphoma Raji and in the melanoma G361. To corroborate this finding, we measured APRIL mRNA expression levels in several tumors and compared them to normal tissues. APRIL mRNA was abundantly detected in thyroid carcinoma and in lymphoma, whereas in the corresponding normal tissues, only weak or no hybridization signals were found (Fig. 2C). In the two other tumors nalyzed by Northern blots (adrenal and paratoid tumors), APRIL mRNA was not elevated. However, *in situ* hybridization revealed abundant APRIL message in human colon adenocarcinoma as compared to normal colon tissue (Fig. 2D).

In order to explore possible activities of APRIL, we expressed a recombinant form of soluble extracellular domain of APRIL (sAPRIL) encompassing amino acids 110 to 250 in 293 cells (9). The full length APRIL gene was amplified from the EST-clone, using a specific 5' forward primer flanked by a EcoRI site (5'-CCAGCCTCATCTCCTTTCTTGC-3') and a specific 3' reverse primer flanked by an XbaI site (5'-TCACAGTTTCACAAACCCCAGG-3'). The amplified fragment was cut with EcoRI/XbaI and cloned into a modified version of pCRIII (Invitrogen), in frame with an N-terminal Flag peptide (15). The soluble form of APRIL (sAPRIL) was generated using the two primers (5'-AAACAGAAGAAGCAGCACTCTG-3') and (5'-TCACAGTTTCACAAACCCCAGG-3') containing a PstI and XbaI site, respectively, and

subsequently cloned into a modified pCRIII vector, containing both a HA signal for protein secretion in eukaryotic cells and an N-terminal Flag epitope (15).

Example 2

The widespread expression of APRIL in tumor cells and tissues suggested to us that APRIL may be associated with tumor growth, and we therefore incubated various tumor cell lines with purified recombinant Flag-tagged sAPRIL (10).

Human embryonic 293T cells, human leukemia Jurkat T-cells, human Burkitt lymphoma B-cells Raji and melanoma cell lines were grown as previously described (16, 17). Other cell lines referred in this paper are deposited in and described by the American Type Culture Collection (Rockville, Maryland). All cell lines were cultured in RPMI or DMEM medium supplemented with 10% fetal calf serum.

Flag-tagged versions of the extracellular domain (residues 103-281) of human FasL and TRAIL (residues 95-281) were recently described (15). Flag-tagged soluble human TWEAK (residues 141-284) was produced in 293 cells (P. S. manuscript in preparation). The anti-Flag antibody M2 were obtained from Kodak International Biotechnologies. An increase in proliferation of the Jurkat T lymphoma cells in the presence of APRIL was observed in a dose dependent manner as detected by an increase in number (approximately 50%) (11) of viable cells 24 hrs after ligand addition (Fig. 3A). The proliferation of cells was detemined by incubating cells at 50,000 cells per well in 100 µl medium with the indicated concentrations of recombinant APRIL, TWEAK, TRAIL, FasL and by determining the number of viable cells using the Celltiter 96 AQ proliferation assay (Promega) after 24 hrs, following the manufacturer's instructions, or by ^{3}H-thymidine incorporation. For the immunodepletion of Flag-APRIL, anti-Flag coupled to agarose was used.

The increase in proliferation was independent of a co-stimulatory signals such as anti-CD3 antibodies or other cytokines. As expected, the addition of identically produced and purified FasL to Jurkat cells decreased the number of viable cells, whereas TWEAK had no effect. The increased cell number correlated with augmented (40%) ^{3}H-thymidine incorporation in APRIL-treated cells (Fig. 3A). Immunodepletion of FLAG-tagged APRIL-containing conditioned medium by anti-FLAG antibodies, but not anti-myc antibodies, reduced the

proliferative effect (Fig. 3B), indicating that the proliferative effect was specific and due to APRIL. Increased proliferation rates were also seen in some B lymphomas (human Raji, mouse A20 cells, but not human BJAB) and on cell lines of epithelial origin such as COS and HeLa, as well as melanomas (Fig. 3C). The breast carcinoma cell MCF-7 did not respond. The effect on Jurkat cells was even more pronounced when the fetal calf serum was reduced from 10% to 1% (Fig. 3D).

Recombinant sAPRIL has forms aggregates which may explain the rather high concentrations needed to detect a proliferative effect with sAPRIL. We therefore transfected NIH-3T3 cells with full-length human APRIL (12) and obtained several APRIL-expressing clones (Fig. 4A). NIH-3T3 APRIL clones were established using the calcium phosphate method of transfection and the full-length FLAG-tagged APRIL containing pCRIII expression vector. Cellular proteins of about 2 x 10^6 cells per lane were electrophoretically separated on a 12% polyacrylamide gel in the presence of SDS under reducing conditions and subsequently transferred to nitrocellulose. Immunoblot analysis of Flag-tagged APRIL was conducted using 5 µg/ml of the rat monoclonal anti-Flag antibody M2 (Kodak International Biotechnologies). First antibodies were detected using affinity purified anti-peroxidase-conjugated donkey anti-mouse antibody (Dianova, Hamburg, Germany) followed by a chemiluminescence reaction using the ECL system (Amersham).

Interestingly, APRIL transfectants proliferated faster than mock-transfectants (Fig. 4B). We reasoned that the APRIL-transfected NIH-3T3 cells might also have a growth advantage *in vivo*. When wild-type or mock-transfected NIH-3T3 cells were injected into nude mice, small palpable tumors were observed after 5-6 weeks (13). In contrast, two clones of NIH-3T3 cells stably transfected with APRIL both induced tumors after only 3-4 weeks. After 6 weeks, mice had to be killed due to the high tumor burden (Fig. 4C). NIH/3T3 fibroblasts (American Type Culture Collection, Rockville, Maryland) and the various transfectants (1 x 10^5 cells) were suspended in 50 µl PBS and injected subcutaneously into the flank region of BALB/c nude mice (Harlan, Zeist, Netherland). Tumor size was measured every three days. Mice were age-matched (3 animals per group).

It will be apparent to those skilled in the art that various modifications and variations can be made in APRIL, compositions and methods of the present invention without departing from

the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

SEQ ID NO:1

```
   1 GGTACGAGGC TTCCTAGAGG GACTGGAACC TAATTCTCCT GAGGCTGAGG
  51 GAGGGTGGAG GGTCTCAAGG CAACGCTGGC CCCACGACGG AGTGCCAGGA
 101 GCACTAACAG TACCCTTAGC TTGCTTTCCT CCTCCCTCCT TTTTATTTTC
 151 AAGTTCCTTT TTATTTCTCC TTGCGTAACA ACCTTCTTCC CTTCTGCACC
 201 ACTGCCCGTA CCCTTACCCG CCCCGCCACC TCCTTGCTAC CCCACTCTTG
 251 AAACCACAGC TGTTGGCAGG GTCCCCAGCT CATGCCAGCC TCATCTCCTT
 301 TCTTGCTAGC CCCCAAAGGG CCTCCAGGCA ACATGGGGGG CCCAGTCAGA
 351 GAGCCGGCAC TCTCAGTTGC CCTCTGGTTG AGTTGGGGGG CAGCTCTGGG
 401 GGCCGTGGCT TGTGCCATGG CTCTGCTGAC CCAACAAACA GAGCTGCAGA
 451 GCCTCAGGAG AGAGGTGAGC CGGCTGCAGG GGACAGGAGG CCCTCCCAG
 501 AATGGGGAAG GGTATCCCTG GCAGAGTCTC CCGGAGCAGA GTTCCGATGC
 551 CCTGGAAGCC TGGGAGAATG GGGAGAGATC CCGGAAAAGG GAGCAGTGC
 601 TCACCCAAAA ACAGAAGAAG CAGCACTCTG TCCTGCACCT GGTTCCCATT
 651 AACGCCACCT CCAAGGATGA CTCCGATGTG ACAGAGGTGA TGTGGCAACC
 701 AGCTCTTAGG CGTGGGAGAG GCCTACAGGC CCAAGGATAT GGTGTCCGAA
 751 TCCAGGATGC TGGAGTTTAT CTGCTGTATA GCCAGGTCCT GTTTCAAGAC
 801 GTGACTTTCA CCATGGGTCA GGTGGTGTCT CGAGAAGGCC AAGGAAGGCA
 851 GGAGACTCTA TTCCGATGTA TAAGAAGTAT GCCCTCCCAC CCGGACCGGG
 901 CCTACAACAG CTGCTATAGC GCAGGTGTCT TCCATTTACA CCAAGGGGAT
 951 ATTCTGAGTG TCATAATTCC CCGGGCAAGG GCGAAACTTA ACCTCTCTCC
1001 ACATGGAACC TTCCTGGGGT TTGTGAAACT GTGATTGTGT TATAAAAAGT
1051 GGCTCCCAGC TTGGAAGACC AGGGTGGGTA CATACTGGAGACAGCCAAGA
1101 GCTGAGTATA TAAAGGAGAG GGAATGTGCA GGAACAGAGGCATCTTCCTG
1151 GGTTTGGCTC CCCGTTCCTC ACTTTTCCCT TTTCATTCCC ACCCCCTAGA
1201 CTTTGATTTT ACGGATATCT TGCTTCTGTT CCCCATGGAG CTCCGAATTC
1251 TTGCGTGTGT GTAGATGAGG GGCGGGGGAC GGGCGCCAGG CATTGTTCAG
1301 ACCTGGTCGG GGCCCACTGG AAGCATCCAG AACAGCACCA CCATCTTA
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SEQ ID NO:2

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   1 MPASSPFLLA PKGPPGNMGG PVREPALSVA LWLSWGAALG AVACAMALLT
  51 QQTELQSLRR EVSRLQGTGG PSQNGEGYPW QSLPEQSSDA LEAWENGERS
 101 RKRRAVLTQK QKKQHSVLHL VPINATSKDD SDVTEVMWQP ALRRGRGLQA
 151 QGYGVRIQDA GVYLLYSQVL FQDVTFTMGQ VVSREGQGRQ ETLFRCIRSM
 201 PSHPDRAYNS CYSAGVFHLH QGDILSVIIP RARAKLNLSP HGTFLGFVKL
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I claim:

1. A DNA sequence encoding APRIL or a fragment thereof.

2. A DNA sequence encoding APRIL said sequence consisting essentially of SEQ. ID. NO.2

3. A DNA sequence consisting essentially of SEQ. ID. NO. 1 said DNA encoding a polypeptide, said polypeptide consisting essentially of SEQ. ID. NO. 2.

4. A DNA sequence that hybridizes to at least a fragment of SEQ. ID NO. 1 said fragment comprising at least 20 consecutive bases, said DNA sequence encoding a polypeptide that is at least 30% homologous with an active site of APRIL.

5. A DNA sequence according to claim 2 wherein said sequence consists essentially of SEQ. ID. NO. 1 with conservative substitutions, alterations or deletions.

6. A recombinant DNA molecule comprising a DNA sequence encoding APRIL said sequence operatively linked to an expression control sequence.

7. The molecule of claim 6 comprising SEQ. ID. NO. 1.

8. A unicellular host transformed with a recombinant DNA molecule of claim 6 or 7.

9. A DNA sequence encoding APRIL having the amino acid sequence of SEQ. ID. NO. 2.

10. A method for producing substantially pure APRIL comprising the step of culturing the unicellular host of claim 8.

11. APRIL essentially free of normally associated animal proteins.

12. The APRIL of claim 11 consisting essentially of SEQ. ID. NO. 2.

13. A pharmaceutical composition comprising a therapeutically effective amount of APRIL or an active fragment thereof, and a pharmaceutically acceptable carrier.

14. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

15. The pharmaceutical composition of claim 13 wherein said APRIL or active fragment thereof comprises SEQ. ID. NO. 2 or a biologically active fragment thereof.

16. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

17. A method for stimulating the immune system comprising administering the composition of claim 13.

18. A method for suppressing the immune system comprising administering an effective amount of the pharmaceutical composition according to claim 13.

19. A method for treating cancer comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 13.

20. A method for identifying a receptor for APRIL comprising:

a. providing APRIL or a fragment thereof,

b. labeling said APRIL or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled of step b.

21. A soluble biologically active fragment of the APRIL of claim 11.

22. A polypeptide comprising an amino acid sequence that is encoded by a DNA selected from the group consisting of:

a. a DNA sequence comprising SEQ. ID. NO. 1;

b. a DNA sequence that hybridizes to the DNA defined in a. and coding on expression for a polypeptide that is at least 40% homologous with the APRIL of claim 12.

23. An antibody preparation that is reactive to APRIL or its receptor or biologically active fragments thereof.

24. The antibody preparation of claim 23 comprising monoclonal antibodies.

25. A method for producing an antibody preparation reactive to APRIL or its receptor comprising the step of immunizing an organism with APRIL or its receptor, or an antigenic fragment thereof.

26. An antisense nucleic acid against APRIL comprising a nucleic acid sequence hybridizing to at least a portion of SEQ. ID. NO. 1.

27. A pharmaceutical composition comprising an antibody preparation according to claim 24.

28. A method of expressing APRIL in a mammalian cell comprising:

a. introducing a gene encoding APRIL into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

29. A method of treating a disorder related to APRIL in a mammal

a. introducing into a cell a therapeutically effective amount of a vector comprising a gene encoding APRIL; and

b. expressing said gene in said mammalian cell.

30. The method of claim 29 wherein the mammal is a human.

31. The method of claim 29 wherein said vector is a virus.

32. A method of inducing cell death comprising the administration of an agent capable of interfering with the binding of APRIL to a receptor.

33. The method of claim 32 further comprising the administration of interferon-γ.

34. A method of treating, suppressing, activating or altering an immune response involving a signaling pathway between APRIL and its receptor, said method comprising the step of administering an effective amount of a blocking agent capable of interfering with the association between APRIL and its receptor.

35. The method of claim 34 wherein said immune response involves human carcinoma cells.

36. A method of treating, suppressing or altering the progression of a cancer comprising adminstering to a patient an effective amount of a blocking agent between April and its receptor capable of interfering with the association.

37. The method of claim 36, wherein the blocking agent is selected from the group consisting of: soluble APRIL, anti-APRIL antibodies, anti-APRIL receptor antibodies, receptor-IG fusions or biologically active fragments thereof.

38. The method of Claim 37 wherein the blocking agent is an anti-APRIL receptor antibody.

39. The method of Claim 36 wherein the blocking agent is administered to a patient in combination with at least one chemotherapeutic agent.

40. The method of claim 39 further comprising the step of administering radiation therapy to said patient.

Novel Ligands of the TNF Family

Abstract of the Disclosure

APRIL, a novel members of the tumor necrosis factor family (TNF), modified APRILs, and pharmaceutical compositions comprising them.

i. Smith et al. 1990; Kohno et al 1990; Loetscher et al 1990; Schall et al 1990.

ii. See Jones et al., 1989; Eck et al., 1992.

iii. K. Tracey, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 255 (1992)); A. Waage, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 275 (1992).

iv. G. D. Roodman, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 117 (1992).

v. A. Nakane, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 285 (1992); I. A. Clark et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 303 (1992); G. E. Grau et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 329 (1992); P-F. Piguet, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 341 (1992); G. H. Wong et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 371 (1992).

vi. S. Malik, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 407 (1992).

vii. D. A. Fox, Am. J. Med.,99, 82 (1995).

viii. D. Goeddel et al., Cold Spring Harbor Symposium Quant. Biol., 51, 597 (1986); G. Trinchieri, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 515 (1992).

ix. L. A. Tartaglia et al., Proc. Natl. Acad. Sci. USA, 88, 9292 (1991); L.A. Tartaglia and D. V. Goeddel, Immunol. Today, 13, 151 (1992).

x. B. Luettig et al., J. Immunol., 143, 4034 (1989); M. Kriegler et al., Cell, 53, 45 (1988).

xi. C. F. Ware et al., in Pathways for Cytolysis, G. M. Griffiths and J. Tschopp (Eds.), Springer-Verlag, Berlin, Heidelberg, p175-218 (1995).

xii. N. Paul et al., Ann. Rev. Immunol.,6,407 (1988).

xiii. P.D. Crowe et al., Science, 264, 707 (1994). (J. Browning et al., Cell, 72, 847 (1993); J. Browning et al., J. Immunol., 154, 33 (1995).

xiv. P. De Togni et al., Science, 264,703 (1993); T.A. Banks et al., J. Immunol., 155, 1685 (1995).

xv. J. Browning and A. Ribolini, J. Immunol.,143,1859 (1989): J. Browning et al., J. Exp. Med.,183, 867 (1996).

xvi. T. Suda et al., J. Immunol., 154, 3806 (1995)(T. Suda et al., J. Immunol., 154, 3806 (1995).

xvii. B.C. Trauth et al., Science, 245,301 (1989); S. Yonehara et al., J. Exp Med., 169,1747 (1989); S. Nagata and P. Goldstein, Science, 267, 1449 (1995); M. H. Falk et al., Blood, 79, 3300 (1992).

xviii. F. Rieux-Laucat et al., Science, 268,1347 (1995); T. Takahashi et al., Cell, 76,969 (1994); R. Watanabe-Fukunaga et al., Nature, 356, 314 (1992).

xix. P. R. Galle and al., J. Exp. Med., 182, 1223 (1995).

xx. F. Silvestris and al., Clin. Immunol. Immunopathol., 75, 197 (1995).

xxi. P.D. Katsikis et al., J. Exp. Med.,181, 2029 (1995); A. D. Badley et al., J. Virol.,70, 199 (1996).

xxii. S. Wiley et al., Immunity, 3, 673 (1995).

xxiii. J. F. Gauchat et al., FEBS Lett., 315, 259 (1993); S. Funakoshi et al., Blood, 83, 2787 (1994).

xxiv. R. C. Allen et al., Science, 259, 990 (1993).

xxv. L. Biancone et al., Kidney-Int.,48, 458 (1995); C. Mohan et al., J. Immunol., 154, 1470 (1995).

xxvi. J. Ruby and al., Nature Medicine, 1, 437 (1995).

xxvii. Z. Wang et al., J. Immunol., 155, 3722 (1995); A. M. Cleary and al., J. Immunol., 155, 3329 (1995).

xxviii. S. Hess and H. Engelman, J. Exp. Med.,183, 159 (1996).

xxix. R. G. Goodwin et al, Cell, 73, 447 (1993); Goodwin et al, Eur. J. Immunol., 23, 2631 (1993); C. A. Smith et al., Cell, 73, 1349 (1993).

xxx. See for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477.

xxxi. See, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815.

33. M.T. Abreu-Martin, A. Vidrich, D.H. Lynch and S.R. Targan. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-" and ligation of Fas ligand. J. Immunol. 155: 4147-4154, 1995.

34. K. Agematsu, T. Kobata, F.-C. Yang, T. Nakazawa, K. Fukushima, M. Kitahara, T. Mori, K. Sugita, C. Morimoto and A. Komiyama. CD27/CD70 interaction directly drives B cell IgG and IgM synthesis. Eur. J. Immunol. 25: 2825-2829, 1995.

35. R. Amakawa, A. Hakem, T.M. Kundig, T. Matsuyama, J.J.L. Simard, E. Timms, A. Wakeham, H.-W. Mittruecker, H. Griesser, H. Takimoto, R. Schmits, A. Shahinian, P.S. Ohashi, J.M. Penninger and T.W. Mak. Impaired negative selection of T cells in Hodgkin=s disease antigen CD30-deficient mice. Cell 84: 551-562, 1996.

36. J.-L. Bodmer, K. Burens, P. Schneider, K. Hofmann, V. Steiner, M. Thome, T. Bornand, M. Hahne, M. Schroeter, K. Becker, A. Wilson, L.E. French, J.L. Browning, H.R. MacDonald, and J. Tschopp. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and fas (apo-1/CD95). Immunity 6: 79-88, 1997.

37. J. Brojatsch, J. Naughton, M.M. Rolls, K. Zingler and J.A.T. Young. Carl, a TNFR-related protein is a cellular receptor for cytopathic avian lleukosis-sarcoma viruses and mediates apoptosis. Cell 87: 845-855, 1996.

38. J.L. Browning, M.J. Androlewicz and C.F. Ware. Lymphotoxin and an associated 33-kDa glycoprotein are expresed on the surface of an activated human T cell hybridoma. J. Immunol. 147: 1230-7, 1991.

39. J.L. Browning, K. Miatkowski, D.A. Griffiths, P.R.Bourdon, C. Hession, C.M. Ambrose and W. Meier. Preparation and characterization of soluble recombinant heterotrimeric complexes of human lymphotoxins alpha and beta. J. Biol. Chem. 271: 8618-26, 1996.

40. J.E. Castro, J.A. Listman, B.A. Jacobson, Y. Wang, P.A. Lopez, S. Ju, P.W. Finn and D.L. Perkins. Fas Modulation of apoptosis during negative selection of thymocytes. Immunity 5: 617-627, 1996.

41. C.-Y.A. Chen and A.-B. Shyu. AU-rich elements: characterization and importance in mRNA degradation. Trends in Biol. Sci. 20: 465-470, 1995.

42. Y. Chicheportiche, C. Ody and P. Vassalli. Identification in mouse macrophages of a new 4 kb mRNA present in hematopoietic tissue which shares a short nucleotide sequence with erythropoietin mRNA. Biochim. Biophys. Res. Comm. 209: 1076-1081, 1995.

43. A.M. Chinnaiyan, K. O=Rourke, G.-L. Yu, R.H. Lyons, M. Garg, D.R. Duan, L. Xing, R. Gentz, J. Ni and V.M. Dixit. Signal transduction by DR3 a death-domain-containing receptor related to TNFR-1 and CD95. Science 274: 990-992, 1996.

44. P. DeTogni et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264: 703-7, 1994.

45. M.A. DeBenedette, N.R. Chu, K.E. Pollok, J. Hurtako, W.F. Wade, B.S. Kwon and T.H.Watts. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP. J. Exp. Med. 181: 985-992, 1995.

46. M. Degli-Esposti, T. Davis-Smith, W.S. Din, P.J. Smolak, R.G. Goodwin and C.A. Smith. Activation of the lymphotoxin-β receptor by cross-linking induces chemokine production and growth arrest in A375 melanoma cells. J. Immunol. 158: 1756-1762, 1997.

47. T.M. Foy, A. Aruffo, J. Bajorath, J.E. Buhlmann and R.J. Noelle. Immune regulation by CD40 and its ligand gp39. Ann. Rev. Immunol. 14: 591-617, 1996.

48. H.J. Gruss, N. Boiani, D.E. Williams, R.J. Armitage, C.A. Smith and R.G. Goodwin. Pleiotropic effects of the CD30 ligand on CD30-expressing cells and lymphoma cell lines. Blood 83: 2045-56, 1994.

49. H.J. Gruss and S.K. Dower. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood 85: 3378-404, 1995.

50. J. Kitson, T. Raven, Y.-P. Jiang, D.V. Goeddel, K.M. Giles, K.-T. Pun, C.J. Grinham, R.Brown and S.N. Farrow. A death domain-containing receptor that mediates apoptosis. Nature 384: 372-375, 1996.

51. S.Y. Lee, C.G. Park and Y. Choi. T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. J. Exp. Med. 183: 669-674, 1996.

52. R.I. Montgomery, M.S. Warner, B.J. Lum and P.G. Spear. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87: 427-436, 1996.

53. S. Nagata. Apoptosis by death factor. Cell 88: 355-365, 1997.

54. R.M. Pitti, S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore and A. Ashkenazi. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 1996.

55. C.A. Smith, T. Farrah and R.G. Goodwin. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76: 959-62, 1994.

56. G.L. Smith. Virus strategies for evasion of the host response to infection. Trends in Microbiol. 3: 81-88, 1994.

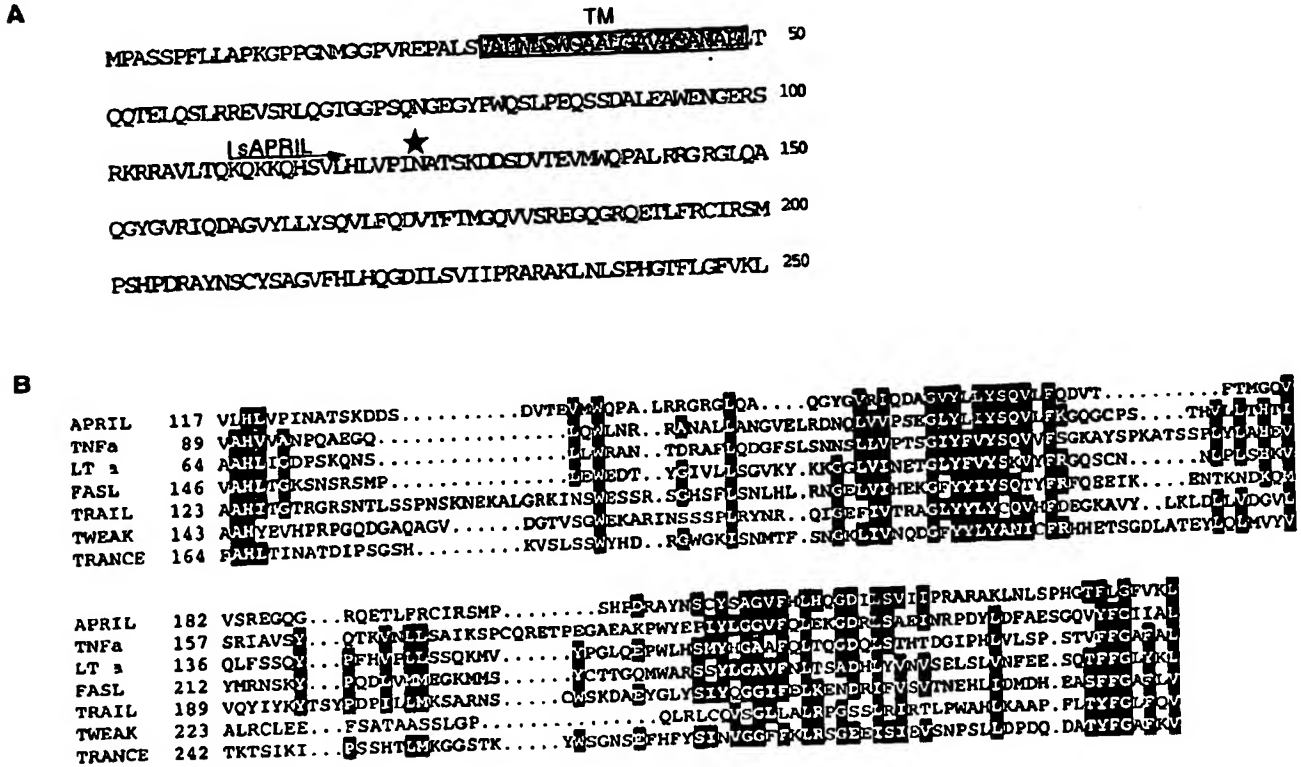
57. E.Strueber and W. Strober. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell independent humoral immune response. J. Exp. Med. 183: 979-989, 1996.

58. H.-K. Sytwu, R.S. Liblau and H.O. McDevitt. The roles of Fas/Apo-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor trangenic mice. Immunity 5: 17-30, 1996.

59. P. Vassalli. The pathophysiology of tumor necrosis factors. Ann. Rev. Immunol. 10: 411-452, 1992.

60. L. Zheng, G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch and M.J. Lenardo. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377: 348-351, 1995.

FIG. 1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150
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200
PSHPDRAYNSCYSAGVFHLHQGDILSVIIPRARAKLNLSPHGTFLGFVKL
250
B
APRIL 117
TNFa 89
LT a 64
FASL 146
TRAIL 123
TWEAK 143
TRANCE 164
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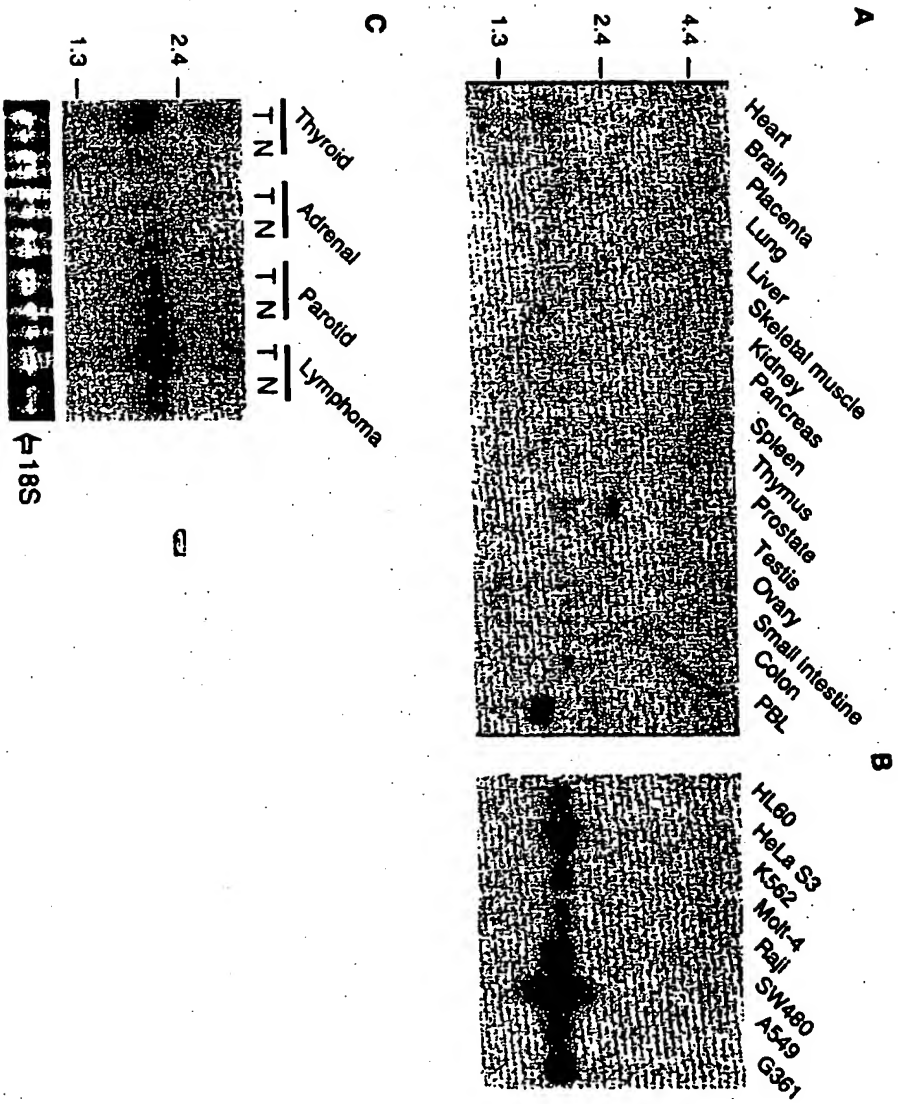
TNFa 157
LT a 136
FASL 212
TRAIL 189
TWEAK 223
TRANCE 242

FIG. 2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1.3
Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Spleen
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Prostate
Testis
Ovary
Small intestine
Colon
PBL
B
HL60
HeLa S3
K562
Molt-4
Raji
SW480
A549
G361
C
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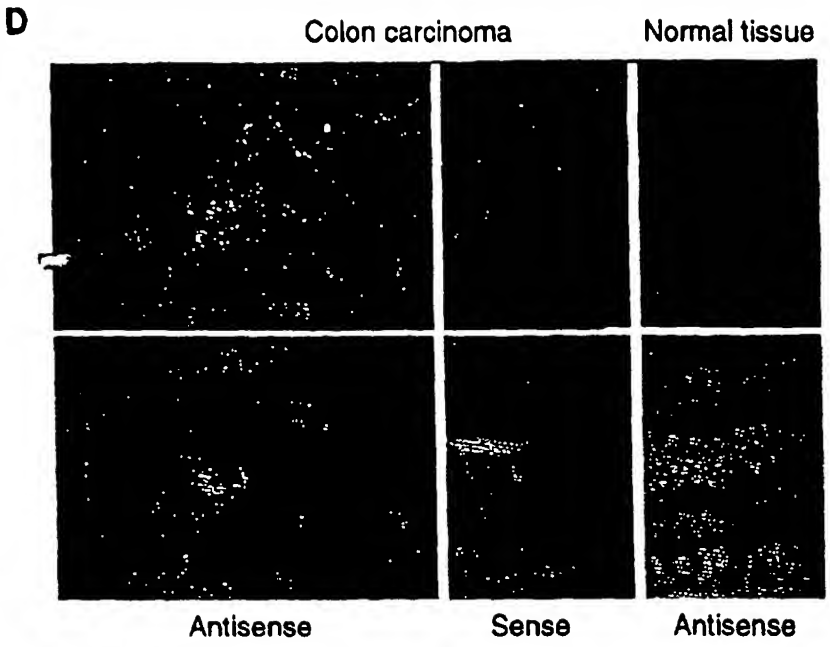
1.3
T N
Thyroid
Adrenal
Parotid
Lymphoma
18S

FIG. 2D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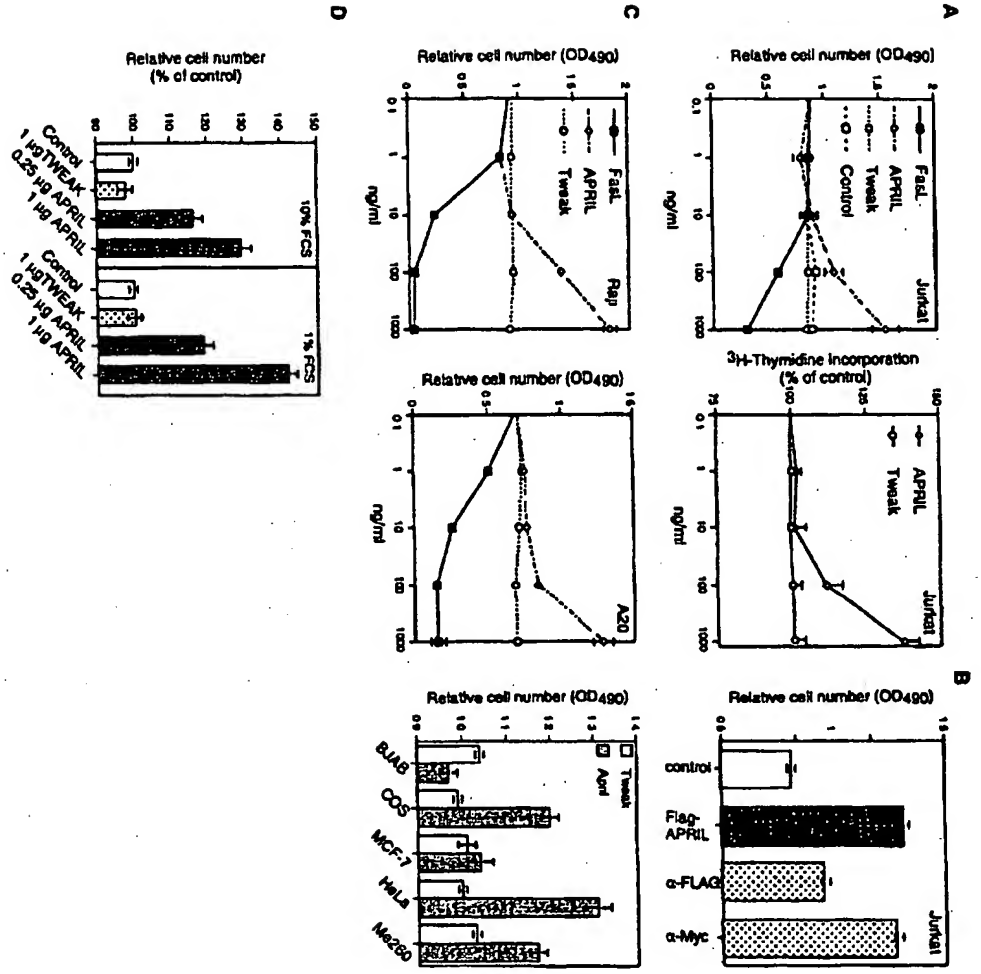
Colon carcinoma
Normal tissue
Antisense
Sense
Antisense

FIG. 13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ng/ml
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Tweak
Control
Jurkat
B
3H-Thymidine incorporation
(% of control)
control
Flag-APRIL
α-FLAG
α-Myc
C
Raji
A20
BJAB
COS
MCF-7
HeLa
Me260
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April
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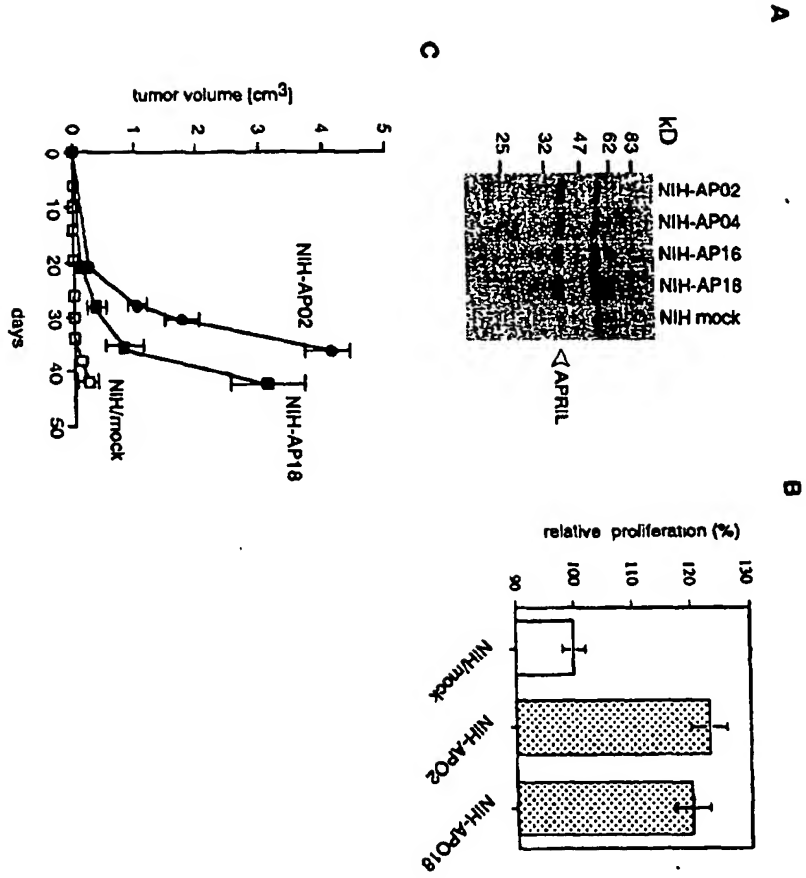
1 μg APRIL

FIG. 4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62
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NIH-AP16
NIH-AP18
NIH mock
APRIL
B
relative proliferation (%)
80
100
110
120
130
NIH/mock
NIH-AP02
NIH-AP18
C
tumor volume [cm3]
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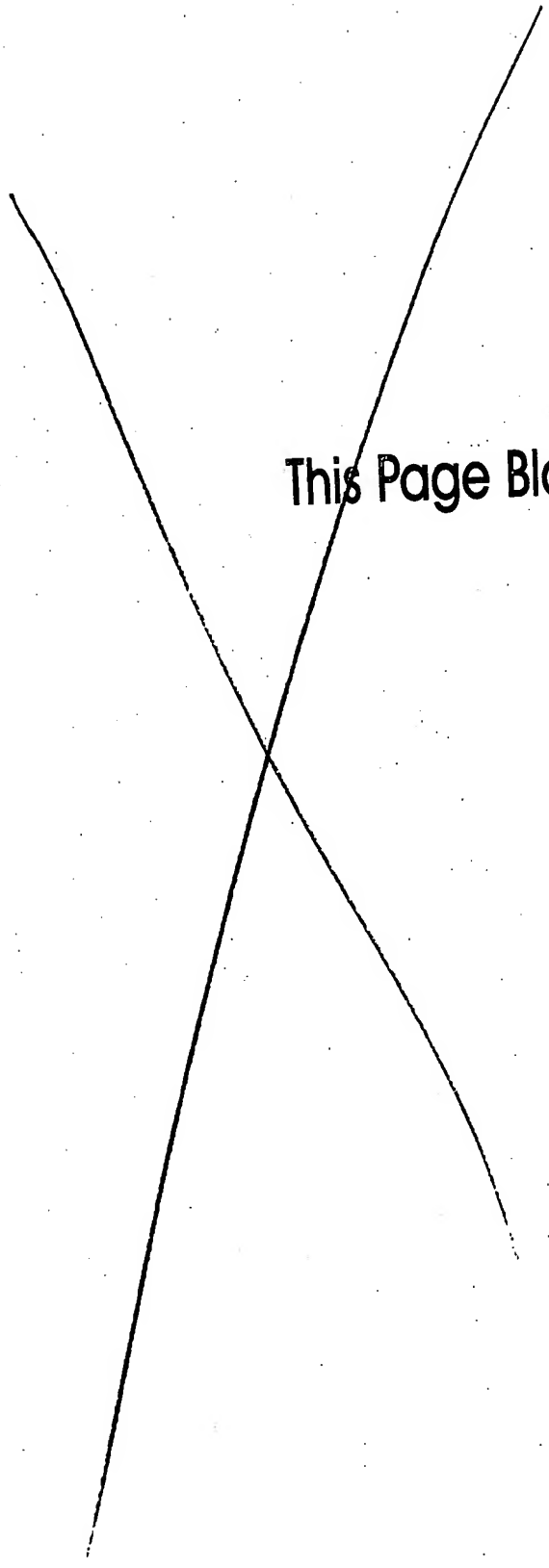
3
4
5
0
10
20
30
40
50
days
NIH-AP02
NIH/mock
NIH-AP18



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Datum/Date
26/03/99

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n° /Patent Nr /Patent No./Brevet n°.
	98946066.2- -PCT/US9819191

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
BIOGEN, INC.

NOTE: The following information concerns the steps which you are required to take for entry into the regional phase before the EPO. You are strongly advised to read it carefully. Failure to take the appropriate steps in due time could lead to the application being deemed withdrawn.

1. European patent application no. 98946066.2 has been allotted to the above-mentioned international patent application.

2. Applicants having neither a residence nor their principal place of business within the territory of one of the EPC Contracting States may initiate the regional (European) processing of the international application themselves, provided they do so before expiry of the 21st or 31st month as from the priority date (see Legal Advice of the EPO no. 18/92 published in OJ EPO 1992, 58).

   Note, however, that such applicants must be represented in the regional phase before the EPO as designated or elected Office by a professional representative whose name appears on the EPO list of representatives (Arts. 133(2) and 134(1) EPC).

   After expiry of the 21st or 31st month, any procedural steps which are taken by the representative of the applicant in the international phase, who is not, however, entitled to practise before the EPO, will have no effect and will, thus, result in loss of rights.

   The appointment of a professional representative entitled to practise before the EPO is possible/advisable at an early stage during the international phase (any time after the 14th month from the priority date) in view of representing applicants before the EPO as designated or elected Office.

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Therefore, an appointment in due time is strongly recommended, if it is intended that this representative should already act for entry into the regional phase, otherwise all communications will be forwarded from the EPO directly to the applicant.

3. Applicants having their address within the territory of one of the EPC Contracting States are not obliged to appoint a professional representative entitled to practise before the EPO to represent them in the regional phase where the EPO is designated or elected Office.

   Note that due to the complexity of the proceedings, applicants are strongly advised to appoint such representative. Please keep in mind that, if a professional representative before the EPO has already acted for the applicant during the international phase, this representative is not automatically regarded as the representative for the regional phase.

4. Applicants and professional representatives are recommended to file EPO Form 1200 (available free of charge from the EPO) for entry into the regional phase. The use of Form 1200, however, is not mandatory.

5. FOR ENTRY INTO THE REGIONAL PHASE BEFORE THE EPO the following procedural steps must be taken. (Note that non-completion or ineffective completion of the required steps will result in loss of rights or other disadvantage.)

   5.1 Within 21 months from the date of filing or (where applicable) from the earliest priority date if the EPO acts as DESIGNATED OFFICE pursuant to Article 22(1) PCT:

   a) Filing of a translation of the international application in an EPO official language if the International Bureau did not publish the application in one of those languages (Art. 22(1) PCT and Rule 104b(1)(a) EPC).
   Note that if such translation is not filed in due time, the international application before the EPO is deemed withdrawn (Art. 24(1)(iii) PCT).

   b) Payment of the national fee national basic fee, the designation fee for each State designated, (where applicable) the claims fees for the eleventh and each subsequent claim] and the search fee, where a supplementary European search report has to be drawn up (Rule 104b(1)(b), (c) EPC).

   Upon expiry of the 21-month time limit provided for in Rule 104b(1) EPC the EPO sends the applicant or his appointed professional representative the communication pursuant to Rule 85a(1) EPC (Form 1217) and (where applicable) Rule 69(1) EPC (Form 1205)

unless it has been notified of its designation as elected Office in due time.

5.2 Within 31 months from the date of filing or (where applicable) from the earliest priority date if the EPO acts as ELECTED OFFICE pursuant to Article 39(1)(a) PCT:

a) Filing of a translation as under 5.1 a).

b) Payment of the fees as under 5.1 b).

c) Filing of the written request for examination and payment of the examination fee (Rule 104b(1)(d) EPC).
Note that both acts must be performed in due time, otherwise the European patent application shall be deemed to be withdrawn (Art. 94(3) EPC).

d) Payment of the renewal fee for the third year, if due before the expiration of the 31-month term (Rule 104b(1)(e) EPC).

6. The amounts of the fees (equivalent in all currencies) are regularly published in the Official Journal of the EPO.

If the national basic fee, the designation fees or the search fee have not been paid in time, they may still be validly paid within a grace period of one month as from notification of an EPO communication (Rule 85a(1) EPC).

If the renewal fee is not paid in time, it may still be validly paid within six months from the due date (Art. 86(2) EPC).

In both cases, a surcharge is due.

7. The international search report under Article 18 PCT (or the declaration under Article 17(2)(a) PCT) has been published by the International Bureau. The date of publication can be ascertained from the copy of the published application documents sent by the International Bureau or from the international search report, if published separately. This publication takes the place of the mention of the publication of the European search report (Art. 157(1) EPC).

A request for examination, comprising a written request and payment of the examination fee, must be filed up to the end of six months after the above date.

However, in view of Article 22 or 39 PCT in conjunction with Rule 104b(1)(d) EPC, the period for filing the request for examination does not expire before 21 or 31 months, respectively, from the date of filing (where applicable, the earliest priority date).

A period of grace of one month from notification of an EPO communication is available in case either or both of the above acts have not been performed in time. Accordingly, a surcharge is due (Rule 85b EPC).

8. This information letter is addressed by the EPO to the agent, if any, having acted for the applicant during the international phase of the application.

   Any further notifications on procedural matters will be addressed to the applicant, respectively his European representative, if the appointment of the latter has been communicated to the EPO in due time.

9. For further details see the information for PCT applicants concerning time limits and procedural steps before the EPO as a designated and as an elected Office under the PCT (published as Supplement No. 1 to OJ EPO 12/1992, with changes published in OJ EPO 1994, 131).

   Concerning the list of professional representatives before the European Patent Office (see points 2 and 3) and EPO Form 1200 (see point 4) we refer to the EPO's Internet address: http://www.european-patent-office.org.

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

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(54) Title: APRIL– A NOVEL PROTEIN WITH GROWTH EFFECTS

(57) Abstract

APRIL, a novel member of the tumor necrosis factor family (TNF), modified APRILs, and pharmaceutical compositions comprising them.

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APRIL- A NOVEL PROTEIN WITH GROWTH EFFECTS

BACKGROUND OF THE INVENTION

The present invention relates to novel ligand and polypeptides which are members of the Tumor Necrosis Factor Family. The novel ligand is designated April for "A Proliferation Inducing Ligand." These proteins or their receptors may have anti-cancer and/or immunoregulatory applications. Furthermore, cells transfected with the genes for these novel ligands may be used in gene therapy to treat tumors, autoimmune and inflammatory diseases or inherited genetic disorders, and blocking antitibodies to these proteins can have immunoregulatory applications.

BACKGROUND OF THE INVENTION

The tumor-necrosis factor (TNF)-related cytokines are mediators of host defense and immune regulation. Members of this family exist in membrane-anchored forms, acting locally through cell-to-cell contact, or as secreted proteins capable of diffusing to more distant targets. A parallel family of receptors signals the presence of these molecules leading to the initiation of cell death or cellular proliferation and differentiation in the target tissue. Presently, the TNF family of ligands and receptors has at least 13 recognized receptor-ligand pairs, including: TNF:TNF-R; LT-α:TNF-R; LT-α/β:LT-β-R; FasL:Fas; CD40L:CD40; CD30L:CD30; CD27L:CD27; OX40L:OX40 and 4-1BBL:4-1BB; trance/rankL: Light and Tweak. The DNA sequences encoding these ligands have only about 25% to about 30% identity in even the most related cases, although the amino acid relatedness is about 50%.

The defining feature of this family of cytokine receptors is found in the cysteine rich extracellular domain initially revealed by the molecular cloning of two distinct TNF receptors.[i] This family of genes encodes glycoproteins characteristic of Type I transmembrane proteins with an extracellular ligand binding domain, a single membrane spanning region and a cytoplasmic region involved in activating cellular functions. The cysteine-rich ligand binding region exhibits a tightly knit disulfide linked core domain, which, depending upon the particular family member, is repeated multiple times. Most receptors have four domains, although there may be as few as three, or as many as six.

Proteins in the TNF family of ligands are characterized by a short N-terminal stretch of normally short hydrophilic amino acids, often containing several lysine or arginine residues thought to serve as stop transfer sequences. Next follows a

transmembrane region and an extracellular region of variable length, that separates the C-terminal receptor binding domain from the membrane. This region is sometimes referred to as the "stalk". The C-terminal binding region comprises the bulk of the protein, and often, but not always, contains glycosylation sites. These genes lack the classic signal sequences characteristic of type I membrane proteins, type II membrane proteins with the C terminal domain lying outside the cell, and a short N-terminus residing in the cytoplasm. In some cases, e.g., TNF and LT-α, cleavage in the stalk region can occur early during protein processing and the ligand is then found primarily in secreted form. Most ligands, however, exist in a membrane form, mediating localized signaling.

The structure of these ligands has been well-defined by crystallographic analyses of TNF, LT-α, and CD40L. TNF and lymphotoxin-α (LT-α) are both structured into a sandwich of two anti-parallel β-pleated sheets with the "jelly roll" or Greek key topology.[ii] The rms deviation between the Cα and β residues is 0.61 C, suggesting a high degree of similarity in their molecular topography. A structural feature emerging from molecular studies of CD40L, TNF and LT-α is the propensity to assemble into oligomeric complexes. Intrinsic to the oligomeric structure is the formation of the receptor binding site at the junction between the neighboring subunits creating a multivalent ligand. The quaternary structures of TNF, CD40L and LT-α have been shown to exist as trimers by analysis of their crystal structures. Many of the amino acids conserved between the different ligands are in stretches of the scaffold β-sheet. It is likely that the basic sandwich structure is preserved in all of these molecules, since portions of these scaffold sequences are conserved across the various family members. The quaternary structure may also be maintained since the subunit conformation is likely to remain similar.

TNF family members can best be described as master switches in the immune system controlling both cell survival and differentiation. Only TNF and LTα are currently recognized as secreted cytokines contrasting with the other predominantly membrane anchored members of the TNF family. While a membrane form of TNF has been well-characterized and is likely to have unique biological roles, secreted TNF functions as a general alarm signaling to cells more distant from the site of the triggering event. Thus TNF secretion can amplify an event leading to the well-described changes in the vasculature lining and the inflammatory state of cells. In contrast, the membrane bound members of the family send signals though the TNF type

receptors only to cells in direct contact. For example T cells provide CD40 mediated "help" only to those B cells brought into direct contact via cognate TCR interactions. Similar cell-cell contact limitations on the ability to induce cell death apply to the well-studied Fas system.

It appears that one can segregate the TNF ligands into three groups based on their ability to induce cell death (Table III). First, TNF, Fas ligand and TRAIL can efficiently induce cell death in many lines and their receptors mostly likely have good canonical death domains. Presumably the ligand to DR-3 (TRAMP/WSL-1) would also all into this category. Next there are those ligands which trigger a weaker death signal limited to few cell types and TWEAK, CD30 ligand and LTα1ß2 are examples of this class . How this group can trigger cell death in the absence of a canonical death domain is an interesting question and suggests that a separate weaker death signaling mechanism exists. Lastly, there are those members that cannot efficiently deliver a death signal. Probably all groups can have antiproliferative effects on some cell types consequent to inducing cell differentiation e.g. CD40 (Funakoshi et al., 1994)

The TNF family has grown dramatically in recent years to encompass at least 11 different signaling pathways involving regulation of the immune system. The widespread expression patterns of TWEAK and TRAIL indicate that there is still more functional variety to be uncovered in this family. This aspect has been especially highlighted recently in the discovery of two receptors that affect the ability of rous sarcoma and herpes simplex virus to replicate as well as the historical observations that TNF has anti-viral activity and pox viruses encode for decoy TNF receptors (Brojatsch et al., 1996; Montgomery et al., 1996; Smith, 1994; Vassalli, 1992). TNF is a mediator of septic shock and cachexia[iii], and is involved in the regulation of hematopoietic cell development.[iv] It appears to play a major role as a mediator of inflammation and defense against bacterial, viral and parasitic infections[v] as well as having antitumor activity.[vi] TNF is also involved in different autoimmune diseases.[vii] TNF may be produced by several types of cells, including macrophages, fibroblasts, T cells and natural killer cells.[viii] TNF binds to two different receptors, each acting through specific intracellular signaling molecules, thus resulting in different effects of TNF.[ix] TNF can exist either as a membrane bound form or as a soluble secreted cytokine.[x]

LT-α shares many activities with TNF, i.e. binding to the TNF receptors,[xi] but unlike TNF, appears to be secreted primarily by activated T cells and some β-lymphoblastoid tumors.[xii] The heteromeric complex of LT-α and LT-β is a membrane

bound complex which binds to the LT-β receptor.[xiii] The LT system (LTs and LT-R) appears to be involved in the development of peripheral lymphoid organs since genetic disruption of LT-β leads to disorganization of T and B cells in the spleen and an absence of lymph nodes.[xiv] The LT-β system is also involved in cell death of some adenocarcinoma cell lines.[xv]

Fas-L, another member of the TNF family, is expressed predominantly on activated T cells.[xvi] It induces the death of cells bearing its receptor, including tumor cells and HIV-infected cells, by a mechanism known as programmed cell death or apoptosis.[xvii] Furthermore, deficiencies in either Fas or Fas-L may lead to lymphoproliferative disorders, confirming the role of the Fas system in the regulation of immune responses.[xviii] The Fas system is also involved in liver damage resulting from hepatitis chronic infection[xix] and in autoimmunity in HIV-infected patients.[xx] The Fas system is also involved in T-cell destruction in HIV patients.[xxi] TRAIL, another member of this family, also seems to be involved in the death of a wide variety of transformed cell lines of diverse origin.[xxii]

CD40-L, another member of the TNF family, is expressed on T cells and induces the regulation of CD40-bearing B cells.[xxiii] Furthermore, alterations in the CD40-L gene result in a disease known as X-linked hyper-IgM syndrome.[xxiv] The CD40 system is also involved in different autoimmune diseases[xxv] and CD40-L is known to have antiviral properties.[xxvi] Although the CD40 system is involved in the rescue of apoptotic B cells,[xxvii] in non-immune cells it induces apoptosis[xxviii]. Many additional lymphocyte members of the TNF family are also involved in costimulation.[xxix]

Generally, the members of the TNF family have fundamental regulatory roles in controlling the immune system and activating acute host defense systems. Given the current progress in manipulating members of the TNF family for therapeutic benefit, it is likely that members of this family may provide unique means to control disease. Some of the ligands of this family can directly induce the apoptotic death of many transformed cells e.g. LT, TNF, Fas ligand and TRAIL (Nagata, 1997). Fas and possibly TNF and CD30 receptor activation can induce cell death in nontransformed lymphocytes which may play an immunoregulatory function (Amakawa et al., 1996; Nagata, 1997; Sytwu et al., 1996; Zheng et al., 1995). In general, death is triggered following the aggregation of death domains which reside on the cytoplasmic side of the TNF receptors. The death domain orchestrates the assembly of various signal

transduction components which result in the activation of the caspase cascade (Nagata, 1997). Some receptors lack canonical death domains, e.g. LTb receptor and CD30 (Browning et al., 1996; Lee et al., 1996) yet can induce cell death, albeit more weakly. It is likely that these receptors function primarily to induce cell differentiation and the death is an aberrant consequence in some transformed cell lines, although this picture is unclear as studies on the CD30 null mouse suggest a death role in negative selection in the thymus (Amakawa et al., 1996). Conversely, signaling through other pathways such as CD40 is required to maintain cell survival. Thus, there is a need to identify and characterize additional molecules which are members of the TNF family thereby providing additional means of controlling disease and manipulating the immune system.

It has been suggested that certain members of the TNF family may provide therapeutic anti-tumor benefits, for example, in combination with IL-2. (See, e.g. U.S. 5,425,940). However, to date, no completely satisfactory treatment for cancer is known. Combination chemotherapy is commonly used in the clinic and in research, for example with antimetabolites, alkylating agents, antibiotics, general poisons, etc. Such drugs are administered alone or in combination in an attempt to obtain a cytotoxic effect on cancers, and/or to reduce or eliminate the emergence of drug-resistant cells, and to reduce side effects.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to a novel polypeptide referred to as APRIL, which substantially obviates one or more of the problems due to the limitations and disadvantages of the related art. The inventors have discovered a new member of the TNF family of cytokines, and defined both the human and murine amino acid sequence of the protein, as well as the DNA sequences encoding these proteins. The claimed invention may be used to identify new diagnostics and therapeutics for numerous diseases and conditions as discussed in more detail below, as well as to obtain information about, and manipulate, the immune system and its processes. Additionally, the invention may be involved in the induction of cell death in carcinomas.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent from the description, or may be learned by practice of the invention. The objectives and other advantages of the

invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof, as well as in the appended drawings.

Thus, to achieve these and other advantages, and in accordance with the purpose of the invention, as embodied and broadly described herein, the invention includes DNA sequences encoding APRIL. Specifically, the invention relates to DNA sequences which encode human APRIL, (SEQ. ID. NO. 1). Additionally, the claimed invention relates to the amino acid sequences of this novel ligand. The amino acid sequence of human APRIL is set forth in SEQ. ID. NO.: 2. Additionally, the inventors have set forth herein the DNA and amino acid sequences for murine APRIL, set forth in SEQ.ID. NOS. 3 and 4 respectively. In other embodiments, the invention relates to sequences that have at least 50% homology with DNA sequences encoding the C terminal receptor binding domain of this ligand and when hybridize to the claimed DNA sequences or fragments thereof, and which encode APRIL having the sequence identified in SEQ. ID. NO. 1 or a protein having similar biological activity.

The invention in certain embodiments furthermore relates to DNA sequences encoding APRIL where the sequences are operatively linked to an expression control sequence. Any suitable expression control sequences are useful in the claimed invention, and can easily be selected by one skilled in the art.

The invention also contemplates recombinant DNAs comprising a sequence encoding APRIL or fragments thereof, as well as hosts with stably integrated APRIL sequences introduced into their genome, or possessing episomal elements. Any suitable host may be used in the invention, and can easily be selected by one skilled in the art without undue experimentation.

In other embodiments, the invention relates to methods of producing substantially pure APRILs comprising the step of culturing transformed hosts. In yet other embodiments, the invention relates to APRIL essentially free of normally associated animal proteins.

The invention encompasses APRIL ligands having the amino acid sequence identified in SEQ. ID. NO. 2, as well as fragments or homologs thereof. In various embodiments, the amino acid and/or the DNA sequences may comprise conservative insertions, deletions and substitutions, as further defined below or may comprise fragments of said sequences.

The invention relates in other embodiments to soluble constructs comprising APRIL, which may be used to directly trigger APRIL mediated pharmacological events. Such events may have useful therapeutic benefits in the stimulation of growth, treatment of cancer, tumors or the manipulation of the immune system to treat immunologic diseases. Soluble forms of the claimed ligands could be genetically reengineered to incorporate an easily recognizable tag, thereby facilitating the identification of the receptors for these ligands.

Additionally, certain embodiments relate to antibodies against APRIL, and their use for the treatment of of cancers, tumors, or manipulation of the immune system to treat immunologic diseases.

In yet other embodiments the invention relates to methods of gene therapy using the genes for APRIL as disclosed and claimed herein.

The pharmaceutical preparations of the invention may, optionally, include pharmaceutically acceptable carriers, adjuvants, fillers, or other pharmaceutical compositions, and may be administered in any of the numerous forms or routes known in the art.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory, and are intended to provide further explanation of the invention as claimed.

The accompanying drawings are included to provide a further understanding of the invention, and are incorporated in, and constitute a part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure legends

Figure 1. (**A**) Predicted amino acid sequence of human APRIL. The predicted transmembrane region (TM, boxed), the potential N-linked glycosylation site (star) and the N-terminus of the recombinant soluble APRIL (sAPRIL) are indicated. (**B**) Comparison of the extracellular protein sequence of APRIL and some members of the TNF ligand family. Identical and homologuous residues are represented in black and shaded boxes, respectively. TNFa, tumor necrosis factor □, LTa, lymphotoxin □, FasL, Fas (CD95) ligand, TRANCE, RANK ligand.

Figure 2. Expression of APRIL (A) Northern blots (2 μg poly A^+ RNA per lane) of various human tissues were probed with APRIL cDNA. (B) APRIL mRNA expression in various tumor cell lines: promyelocitic leukemia HL 60 ; HeLa Cell S3; chronic myelogenous leukemia K562; lymphoblastic leukemia Molt-4; Burkitt's lymphoma Raji; colorectal adenocarcinoma A459; melanoma G361. (C) APRIL mRNA expression in four different human tumors (T) and normal tissues (N). The 18S rRNA band shows equal loading. (D) APRIL mRNA expression in primary colon carcinoma. In situ hybridization revealed abundant APRIL message in human colon carcinoma as compared to normal colon tissue. Colon tumor tissue sections and adjacent normal colon tissue were hybridized to antisense APRIL ^{35}S-labeled cRNA, and as control, colon tumor tissue sections were also hybridized to sense APRIL ^{35}S cRNA (negative control). The upper panels are dark field micrographs, the lower panels are the corresponding light field micrographs.

Figure 3. APRIL stimulates cell growth. (A) Dose dependent increase of proliferation of Jurkat (human leukemia T cells), as determined 24 hrs after addition of soluble APRIL. Controls are Fas ligand (FasL), TWEAK and no ligand (Control) (left panel, cell viability; right panel, ^3H-Thymidine incorperation). (B) Influence of immunodepletion of FLAG-tagged APRIL on tumor cell growth. The proliferative effect of FLAG-tagged APRIL is neutralized by anti-FLAG antibodies, but not by anti-myc antibodies. (C) Effect of APRIL on the proliferation rate of Raji (human Burkitt lymphoma B cells), A20 cells (mouse B lymphoma), BJAB (human B lymphoma), COS (canine epithelial cells), MCF-7 (human breast adenocarcinoma), HeLa (human epitheloid carcinoma) and ME260 (human melanoma). (D) Influence of fetal calf serum concentration on APRIL-induced proliferation of Jurkat cells.

Figure 4. APRIL accelerates tumor growth. (A) Characterization of APRIL-transfected NIH-3T3 clones. FLAG-APRIL levels of the various clones were analyzed by Western blotting using an anti-FLAG antibody. The arrow points to the APRIL protein, the high molecular weight protein is detected non-specifically (B) APRIL-expressing NIH-3T3 clones grow faster than mock-transfeced clones. (C) Increased

tumor growth of APRIL-expressing NIH-3T3 clones. NIH-3T3 cells (1×10^5 cells) and APRIL (NIH-AP, 2 different clones) transfectants (1×10^6 cells) were injected subcutaneously into nude mice, and tumor growth monitored.

Figure 5. An alignment of the human and mouse APRIL amino acid sequences showing the extensive identity between the two proteins. Identical residues are marked with the overlaying dot. The underlined residues represent a potential N-linked glycosylation site. The intiating methionine is considered a likely start site, however, it is possible that in frame methionines further upstream may serve as the actual start site, for example, in the human sequence.

DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention. This invention relates to DNA sequences that code for human or mouse APRIL, fragments and homologs thereof, and expression of those DNA sequences in hosts transformed with them. The invention relates to uses of these DNA sequences and the peptides encoded by them. Additionally, the invention encompasses both human and mouse amino acid sequences for and APRIL, or fragments thereof, as well as pharmaceutical compositions comprising or derived from them. The invention relates to methods of stimulating cell growth with APRIL, or, alternatively, methods of inhibiting tumorogenesis using antibodies directed against APRIL or a receptor of APRIL.

A. DEFINITIONS

"Homologous", as used herein, refers to the sequence similarity between sequences of molecules being compared. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

As used herein, the term "cancer" refers to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer, mammary adenocarcinoma, pharyngeal squamous cell carcinoma, and gastrointestinal or stomach cancer. Preferably, the cancer is leukemia, mastocytoma, melanoma, lymphoma, mammary adenocarcinoma, and pharyngeal squamous cell carcinoma

A "purified preparation" or a "substantially pure preparation" of a polypeptide, as used herein, means a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from other substances, e.g., antibodies, matrices, etc., which are used to purify it.

"Transformed host" as used herein is meant to encompass any host with stably integrated sequence, i.e. a sequence encoding APRIL, introduced into its genome.

A "treatment", as used herein, includes any therapeutic treatment, e.g., the administration of a therapeutic agent or substance, e.g., a drug.

A "substantially pure nucleic acid", e.g., a substantially pure DNA, is a nucleic acid which is one or both of: (1) not immediately contiguous with either one or both of the sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or (2) which is substantially free of a nucleic acid sequence with which it occurs in the organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding APRIL.

The terms "peptides", "proteins", and "polypeptides" are used interchangeably herein.

"Biologically active" as used herein, means having an in vivo or in vitro activity which may be performed directly or indirectly. Biologically active fragments of APRIL may have, for example, 70% amino acid homology with the active site of

APRIL, more preferably at least 80%, and most preferably, at least 90% homology. Identity or homology with respect to APRIL is defined herein as the percentage of amino acid residues in the candidate sequence which are identical to the APRIL residues in SEQ. ID. NOS. 2 or 4.

"Ligand" as used herein generically refers to APRIL. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature.

Introduction:

APRIL, a novel member of the TNF family, is described in detail herein. The inventors have found that while transcript of APRIL are of low abundance in normal tissues, high levels of mRNA are detected in several tumor cell lines, as well as in colon carcinomas, metastatic lymphomas and thyroid tumors. In vitro, the addition of recombinant APRIL stimulates the proliferation of various cell lines. Moreover, transfection of APRIL into NIH-3T3 cells dramatically accelerated tumor growth in nude mice when compared to mock transfectants. The expression and growth stimulating effect of APRIL on tumor cells in vitro and in vivo suggests that APRIL is implicated in tumorigenesis.

APRIL appears to be unique among the members of the TNF family as it is both abundantly expressed in tumor cells and stimulates growth of many different tumor cell lines given the apparent role of APRIL is tumorigenesis, the antagonistic antibodies to APRIL, or the APRIL receptor, will provide novel approaches to cancer treatment.

B. DNA SEQUENCES OF THE INVENTION

As described herein, one aspect of the invention features a substantially pure (or recombinant) nucleic acid which includes a nucleotide sequence encoding APRIL, such as the DNA described in SEQ. ID. NO. 1 and/or equivalents of such nucleic acids. The term nucleic acid as used herein can include fragments and equivalents, such as, for example, sequences encoding functionally equivalent peptides. Equivalent nucleotide sequences may include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, mutations, etc. and include sequences

that differ from the nucleotide sequence encoding APRIL shown in SEQ. ID NO: 1 due to the degeneracy of the genetic code.

The invention will be described generally by reference to the human sequences, although one skilled in the art will understand that the mouse sequences or sequences encoding APRIL from other species having a high level of homology with human, and are encompassed herein. The human proteins appear to have all of the characteristics of the TNF family, i.e., a type II membrane protein organization and conservation of the sequence motifs involved in the folding of the protein into the TNF anti-parallel β-sheet structure.

The sequences of the invention can be used to prepare a series of DNA probes that are useful in screening various collections of natural and synthetic DNAs for the presence of DNA sequences that are closely related to APRIL, or fragments or derivatives thereof. One skilled in the art will recognize that reference to APRIL as used herein, refers also to biologically active derivatives, fragments or homologs thereof.

The DNA sequences of the invention coding on APRIL can be employed to produce the claimed peptides on expression in various prokaryotic and eukaryotic hosts transformed with them. These peptides may be used in anti-cancer, and immunoregulatory applications. In general, this comprises the steps of culturing a host transformed with a DNA molecule containing the sequence encoding APRIL, operatively-linked to an expression control sequence.

The DNA sequences and recombinant DNA molecules of the present invention can be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal or synthetic DNA sequences. The expression vectors of the invention are characterized by at least one expression control sequence that may be operatively linked to the APRIL DNA sequence inserted in the vector, in order to control and to regulate the expression of the DNA sequence.

Furthermore, within each expression vector, various sites may be selected for insertion of a sequence of the invention. The sites are usually designated by a restriction endonuclease which cuts them, and these sites and endonucleases are well recognized by those skilled in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the desired DNA fragment. Instead, the vector may be cloned to the

fragment by alternate means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment, and its operative linking therein to an expression control sequence, is determined by a variety of factors. These factors include, but are not limited to, the size of the protein to be expressed, the susceptibility of the desired protein to proteolytic degradation by host cell enzymes, number of sites susceptible to a particular restriction enzyme, contamination or binding of the protein to be expressed by host cell proteins which may prove difficult to remove during purification. Additional factors which may be considered include expression characteristics such as the location of start and stop codons relative to the vector sequences, and other factors which will be recognized by those skilled in the art. The choice of a vector and insertion site for the claimed DNA sequences is determined by a balancing of these factors, not all selections being equally effective for a desired application. However, it is routine for one skilled in the art to analyze these parameters and choose an appropriate system depending on the particular application.

One skilled in the art can readily make appropriate modifications to the expression control sequences to obtain higher levels of protein expression, i.e. by substitution of codons, or selecting codons for particular amino acids that are preferentially used by particular organisms, to minimize proteolysis or to alter glycosylation composition. Likewise, cysteines may be changed to other amino acids to simplify production, refolding or stability problems.

Thus, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention. However, a particular selection of a host/expression vector combination may be made by those of skill in the art. Factors one may consider include, for example, the compatibility of the host and vector, toxicity to the host of the proteins encoded by the DNA sequence, ease of recovery of the desired protein, expression characteristics of the DNA sequences and expression control sequences operatively linked to them, biosafety, costs and the folding, form or other necessary post-expression modifications of the desired protein.

APRIL produced by hosts transformed with the sequences of the invention, as well as native APRIL purified by the processes of this invention, or produced from the claimed amino acid sequences, are useful in a variety of compositions and methods for anticancer, antitumor and immunoregulatory applications. They are also useful in therapy and methods directed to other diseases.

This invention also relates to the use of the DNA sequences disclosed herein to express APRIL under abnormal conditions, i.e. in a gene therapy setting. Additionally, APRIL may be expressed in tumor cells under the direction of promoters appropriate for such applications. Such expression could enhance anti-tumor immune responses or directly affect the survival of the tumor. APRIL is also likely to affect the survival of an organ graft by altering the local immune response. In this case, the graft itself or the surrounding cells would be modified with an engineered gene encoding APRIL.

Another aspect of the invention relates to the use of the isolated nucleic acid encoding either APRIL in "antigens" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular conditions with the cellular mRNA and/or DNA encoding the APRIL sequence of interest, so as to inhibit expression of the encoded protein, i.e. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to a range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA which is complementary to at least a portion of the cellular mRNA which encodes APRIL. Alternatively, the antisense construct can be an oligonucleotide probe which is generated ex vivo. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, and are therefor stable in vivo. Exemplary nucleic acids molecules for use as antisense oligonucleotides are phosphoramidates, phosphothioate and methylphosphonate analogs of DNA (See, e.g., 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van Der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48: 2659-2668, specifically incorporated herein by reference.

C. APRIL AND AMINO ACID SEQUENCES THEREFOR

APRIL, as discussed above, is a member of the TNF family. The protein, fragments or homologs of APRIL may have wide therapeutic and diagnostic applications as discussed in more detail below.

Although the precise three dimensional structure of APRIL is not known, it is predicted that, as a member of the TNF family, it may share certain structural characteristics with other members of the family.

Comparison of the claimed APRIL sequence with other members of the human TNF family reveals considerable structural similarity. All the proteins share several regions of sequence conservation in the extracellular domain. The overall sequence homology of the extracellular domain of APRIL show the highest homology to FasL (21% amino acid identities), TNFα (20%), LT-β (18%), followed by TRAIL, TWEAK and TRANCE (15%). Figure 2.

The novel polypeptides of the invention specifically interact with a receptor, which has not yet been identified. However, the peptides and methods disclosed herein enable the identification of receptors which specifically interact with APRIL or fragments thereof.

The claimed invention in certain embodiments includes peptides derived from APRIL which have the ability to bind to its receptors. Fragments of APRIL can be produced in several ways, e.g., recombinantly, by PCR, proteolytic digestion or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end or both ends of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments.

Polypeptide fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f- moc or t-boc chemistry. For example, peptides and DNA sequences of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragment, or divided into overlapping fragments of a desired length. Methods such as these are described in more detail below.

D. Generation of Soluble Forms of APRIL

Soluble forms of APRIL can often signal effectively and hence can be administered as a drug which now mimics the natural membrane form. It is possible that APRIL as claimed herein is naturally secreted as a soluble cytokines, however, if not, one can reengineer the gene to force secretion. To create a soluble secreted form

of APRIL, one would remove at the DNA level the N-terminus transmembrane regions, and some portion of the stalk region, and replace them with a type I leader or alternatively a type II leader sequence that will allow efficient proteolytic cleavage in the chosen expression system. A skilled artisan could vary the amount of the stalk region retained in the secretion expression construct to optimize both receptor binding properties and secretion efficiency. For example, the constructs containing all possible stalk lengths, i.e. N-terminal truncations, could be prepared such that proteins starting at amino acids 81 to 139 would result. The optimal length stalk sequence would result from this type of analysis.

E. Generation of Antibodies Reactive with APRIL

The invention also includes antibodies specifically reactive with APRIL or its receptor. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers, or other techniques are well known in the art.

An immunogenic portion of APRIL or its receptor can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of APRIL, or its receptor, e.g. antigenic determinants of a polypeptide of SEQ. ID. NO.: 2, or a closely related human or non-human mammalian homolog (e.g. 70, 80 or 90 percent homologous, more preferably at least 95 percent homologous). In yet a further preferred embodiment of the present invention, the anti-APRIL or anti-APRIL-receptor antibodies do not substantially cross react (i.e. react specifically) with a protein which is e.g., less than 80 percent homologous to SEQ. ID. NO. 2; preferably less than 90 percent homologous with SEQ. ID. NO.: 2; and, most preferably less than 95 percent homologous with SEQ. ID. NO.: 2. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is less than 10 percent, more preferably less than 5 percent, and even more preferably less than 1 percent, of the binding affinity for a protein of SEQ. ID. NO. 2.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with APRIL, or its receptor. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibodies of the present invention are further intended to include biospecific and chimeric molecules having anti-APRIL or anti-APRIL -receptor activity. Thus, both monoclonal and polyclonal antibodies (Ab) directed against APRIL and its receptor, and antibody fragments such as Fab' and $F(ab')_2$, can be used to block the action of APRIL and its respective receptor.

Various forms of antibodies can also be made using standard recombinant DNA techniques. (Winter and Milstein, Nature 349: 293-299 (1991) specifically incorporated by reference herein.) For example, chimeric antibodies can be constructed in which the antigen binding domain from an animal antibody is linked to a human constant domain (e.g. Cabilly et al., U.S. 4,816,567, incorporated herein by reference). Chimeric antibodies may reduce the observed immunogenic responses elicited by animal antibodies when used in human clinical treatments.

In addition, recombinant "humanized antibodies" which recognize APRIL, or its receptor can be synthesized. Humanized antibodies are chimeras comprising mostly human IgG sequences into which the regions responsible for specific antigen-binding have been inserted. Animals are immunized with the desired antigen, the corresponding antibodies are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized antibodies minimize the use of heterologous (i.e. inter species) sequences in human antibodies, and thus are less likely to elicit immune responses in the treated subject.

Construction of different classes of recombinant antibodies can also be accomplished by making chimeric or humanized antibodies comprising variable domains and human constant domains (CH1, CH2, CH3) isolated from different classes of immunoglobulins. For example, antibodies with increased antigen binding site valencies can be recombinantly produced by cloning the antigen binding site into

vectors carrying the human chain constant regions. (Arulanandam et al., J. Exp. Med., 177: 1439-1450 (1993), incorporated herein by reference.)

In addition, standard recombinant DNA techniques can be used to alter the binding affinities of recombinant antibodies with their antigens by altering amino acid residues in the vicinity of the antigen binding sites. The antigen binding affinity of a humanized antibody can be increased by mutagenesis based on molecular modeling. (Queen et al., Proc. Natl. Acad. Sci. 86: 10029-33 (1989) incorporated herein by reference.

F. Generation of Analogs: Production of Altered DNA and Peptide Sequences

Analogs of APRIL can differ from the naturally occurring Ligands in amino acid sequence, or in ways that do not involve sequence, or both. Non-sequence modifications include in vivo or in vitro chemical derivatization of APRIL. Non-sequence modifications include, but are not limited to, changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

Preferred analogs include, APRIL or biologically active fragments thereof, whose sequences differ from the sequence given in SEQ. ID NO. 2, by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity of APRIL. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g. substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and, phenylalanine, tyrosine.

TABLE 1

CONSERVATIVE AMINO ACID REPLACEMENTS

for amino Acid	code	replace with any of:
Alanine	A	D-Ala, Gly, Beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met

Lysine	K	D-Lys, Arg, D-Arg, Homo-arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3, 4 or 5-phenylproline, cis-3, 4, or 5-phenylproline
Proline	P	D-Pro, L-I-thoazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Useful methods for mutagenesis include PCR mutagenesis and saturation mutagenesis as discussed in more detail below. A library of random amino acid

sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences.

-PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity can be used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized can be amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments can be inserted into appropriate cloning vectors to provide random mutant libraries.

-Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro,* and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as of a protein can be prepared by random mutagenesis of DNA which those that alter function, can be obtained. The distribution of point mutations is not biased toward conserved sequence elements.

-Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art[xxx] Such techniques have been employed in the directed evolution of other proteins[xxxi].

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and

then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

-Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989) specifically incorporated by reference. In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions can then be refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

-Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983) incorporated herein by reference. Briefly, the desired DNA can be altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that

described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]) incorporated herein by reference.

-Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]) incorporated herein by reference. The starting material can be a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

-Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the

resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, binding to APRIL or its receptor, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

The invention also provides for reduction of the protein binding domains of the claimed polypeptides or their receptors, to generate mimetics, e.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of APRIL with its respective receptor. The critical residues of the APRIL involved in molecular recognition of a receptor polypeptide or of a downstream intracellular protein, can be determined and used to generate APRIL or its receptor-derived peptidomimetics which competitively or noncompetitively inhibit binding of APRIL with a receptor. (see, for example, "Peptide inhibitors of human papilloma virus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A), specifically incorporated herein by reference.

By making available purified and recombinant APRIL, the present invention provides assays which can be used to screen for drug candidates which are either agonists or antagonists of the normal cellular function, in this case, of APRIL or its receptor. In one embodiment, the assay evaluates the ability of a compound to modulate binding between APRIL and a receptor. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by the skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

Isolation of a receptor binding to APRIL

Ligands of the TNF family can be used to identify and clone receptors. With the described APRIL sequences, one could fuse the 5' end of the extracellular domain which constitutes the receptor binding sequence to a marker or tagging sequence and then add a leader sequence that will force secretion of APRIL in any of a number of expression systems. One example of this technology is described by Browning et al., (1996) (JBC 271, 8618-8626) where the LT-β ligand was secreted in such a form. The VCAM leader sequence was coupled to a short myc peptide tag followed by the extracellular domain of the LT-β. The VCAM sequence is used to force secretion of the normally membrane bound LT-β molecule. The secreted protein retains a myc tag on the N-terminus which does not impair the ability to bind to a receptor. Such a secreted protein can be expressed in either transiently transfected Cos cells or a similar system, e.g., EBNA derived vectors, insect cell/baculovirus, picchia etc. The unpurified cell supernatant can be used as a source of the tagged ligand.

Cells expressing the receptor can be identified by exposing them to the tagged ligand. Cells with bound ligand are identified in a FACS experiment by labeling the myc tag with an anti-myc peptide antibody (9E10) followed by phycoerythrin (or a similar label) labeled anti-mouse immunoglobulin. FACS positive cells can be readily identified and would serve as a source of RNA encoding for the receptor. An expression library would then be prepared from this RNA via standard techniques and separated into pools. Pools of clones would be transfected into a suitable host cell and binding of the tagged ligand to receptor positive transfected cells determined via microscopic examination, following labeling of bound myc peptide tag with an enzyme labeled anti-mouse Ig reagent, i.e. galactosidase, alkaline phosphatase or luciferase labeled antibody. Once a positive pool has been identified, the pool size would be reduced until the receptor encoding cDNA is identified. This procedure could be carried out with either the mouse or human APRIL, as one may more readily lead to a receptor.

G. METHODS OF TREATMENT AND PHARMACEUTICAL COMPOSITIONS

The methods of the invention for the treatment of cancers involve the administration to a patient, preferably a mammalian host, such as a dog, cat, or human, an effective amount of a claimed composition comprising a blocking agent capable of interfering with the association between APRIL and its receptor. Such blocking agents include, but are not limited to soluble APRIL, anti-APRIL antibodies, anti-APRIL receptor antibodies, or biologically active fragments thereof. Additionally, an inhibitory form of APRIL can be made by mutating APRIL, while maintaining the ability to block the association between APRIL and its receptor. Blocking agents may preferably comprise a receptor IG fusion protein, which can be constructed by methods known to those of skill in the art.

The methods of the invention are useful for treating all cancers, including, but not limited to, cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancerm mammary adenocarcinoma, pharyngeal squamous cell carcinoma, and gastrointestinal or stomach cancer. Additionally, such blocking agents are useful for the treatment of proliferative conditions that are not considered to be tumors, i.e. cellular hyperproliferation (hyperplasia), such as, for example, scleroderma, pannus formation in rheumatoid arthritis, postsurgical scarring and lung, liver and uterine fibrosis.

Pharmaceutical compositions of the invention may comprise a therapeutically effective amount of APRIL, or its receptor, or fragments or mimetics thereof, and, optionally may include pharmaceutically acceptable carriers. Accordingly, this invention provides methods for treatment of cancer, and methods of stimulating, or in certain instances, inhibiting the immune system, or parts thereof by administering a pharmaceutically effective amount of a compound of the invention or its pharmaceutically acceptable salts or derivatives. It should of course by understood that the compositions and methods of this invention can be used in combination with other therapies for various treatments.

The compositions can be formulated for a variety of routes of administration, including systemic, topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the compositions of the invention can be formulated in

liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compositions may be formulated in solid form and, optionally, redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

The compositions can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration, bile salts, fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the compositions are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams as known in the art.

The dose and dosing regimen will depend on the type of cancer, the patient and the patient's history. The amount must be effective to treat, suppress, or alter the progression of cancer. The doses may be single doses or multiple doses. If multiple doses are employed, as preferred, the freuence of administration will depend, for rexample, on the type of host and and type of cancer, dosage amounts etc. For some types of cancers or cancer lines, dily administration wil be effective, whereas for others, administration every other day or every third day will be effective. The amount of active compound administered at one time or over the course of treatment will depend on many factors. For example, the age and size of the subject, the severity and course of the disease being treated, the manner and form of administration, and the judgments of the treating physician. However, an effective dose may be in the range of from about 0.005 to about 5 mg/kg/day, preferably about 0.05 to about 0.5 mg/kg/day. The dosage amount which will be most effective will be one which results in no tumor appearance or complete regression of the tumor, and is not toxic to the patient. One skilled in the art will recognize that lower and higher doses may also be useful.

Gene constructs according to the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of APRIL.

Expression constructs of the APRIL can be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the gene for APRIL to cells in vivo. Approaches include insertion of the gene in viral

vectors which can transfect cells directly, or delivering plasmid DNA with the help of, for example, liposomes, or intracellular carriers, as well as direct injection of the gene construct. Viral vector transfer methods are preferred.

A pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA, RNA or amino acid sequences to which they specifically bind. In other aspects, the claimed invention may be used to evaluate a chemical entity for its ability to interact with, e.g., bind or physically associate with APRIL or a fragment thereof. The method includes contacting the chemical entity with APRIL, and evaluating the ability of the entity to interact with APRIL. Additionally, APRIL can be used in methods of evaluating naturally occurring APRIL or receptors of APRIL, as well as to evaluate chemical entities which associate or bind with receptors of APRIL. It may be desirable to use tagged versions of APRIL to facilitate the detection of APRIL binding to its receptor, or receptor positive cells, such as, for example, the purpose of screening for agents that block the APRIL ligand-APRIL receptor interaction. Additionally, one may use APRIL transfected cell lines that have increased growth rates as the basis for screening assays for molecules that block APRIL activity.

In certain aspects, the claimed invention features a method for evaluating a chemical entity for the ability to modulate the interaction between APRIL and its respective receptor. The method includes combining a receptor for APRIL, and APRIL under conditions wherein the pair is capable of interacting, adding the chemical entity to be evaluated and detecting the formation or dissolution of complexes. These modulating agents may be further evaluated in vitro, e.g. by testing its activity in a cell free system, and then, optionally administering the compound to a cell or animal, and evaluating the effect.

I. EXAMPLES

Example 1

Northern blot analysis of APRIL revealed that the expression of APRIL was weak and restricted only to a few tissues (Fig. 2A). Two transcripts of 2.1 kb and 2.4 kb were found in the prostate, whereas PBLs revealed a shorter transcript of 1.8 kb. Northern blot analysis was performed by using Human Multiple Tissue Northern Blots I and II (Clontech #7760-1 and #7759-1), Human Cancer Cell Line MTN Blot (Clontech #7757-1) and Human Tumor Panel Blot V (Invitrogen D3500-01). The membranes were incubated in ExpressHyb hybridization solution (Clontech #8015-1) for at least 1 hour at 62°C. The random-primed cDNA probe (Boehringer Mannheim) was synthesized using cDNA corresponding to the extracellular domain of APRIL as template. The heat-denatured cDNA probe was added at 1.5 $x10^6$ cpm/ml in fresh ExpressHyb. The membrane was hybridized 12-24 hr at 62°C, washed three times in 2xSSC containing 0.05% SDS and exposed at -70°C. Northern blot analysis of APRIL revealed that the expression of APRIL was weak and restricted only to a few tissues. Two transcripts of 2.1 kb and 2.4 kb were found in th eprostrate, whereas PBLs revealed a shorter transcript of 1.8 kb.

A longer exposure time revealed the 2.1 kb APRIL mRNA in colon, spleen, and pancreas (data not shown). This restricted distribution of the APRIL mRNA is consistent with the origin of cDNA clones currently available in the EST database. Of the 23 clones identified only two were derived from normal tissues (pregnant uterus, pancreatic islands). Remarkably, the remainder of the EST-clones (21 clones, 91%) were present in cDNA libraries generated from tumors or tumor-derived cell lines (Ovary tumor, 11; prostate tumor, 3; Gessler Wilms tumor, 1; colon carcinoma, 1; endometrial tumor, 1; parathyroid tumors, 1; pancreas tumor, 1; T-cell lymphoma, 1; LNCAP adenocarcinoma derived cell line, 1). This prompted us to test transformed cell lines for the expression of APRIL mRNA (Figure 2B), and indeed, all cell lines strongly expressed the 2.1 kb transcript of APRIL.

Highest APRIL-specific signals were detected in the colorectal adenocarcinoma SW480, the Burkitt's lymphoma Raji and in the melanoma G361. To corroborate this finding, we measured APRIL mRNA expression levels in several tumors and compared them to normal tissues. APRIL mRNA was abundantly detected in thyroid carcinoma and in lymphoma, whereas in the corresponding normal tissues,

only weak or no hybridization signals were found (Fig. 2C). In the two other tumors nalyzed by Northern blots (adrenal and paratoid tumors), APRIL mRNA was not elevated. However, *in situ* hybridization revealed abundant APRIL message in human colon adenocarcinoma as compared to normal colon tissue (Fig. 2D).

In order to explore possible activities of APRIL, we expressed a recombinant form of soluble extracellular domain of APRIL (sAPRIL) encompassing amino acids 110 to 250 in 293 cells (9). The full length APRIL gene was amplified from the EST-clone, using a specific 5' forward primer flanked by a EcoRI site (5'-CCAGCCTCATCTCCTTTCTTGC-3') and a specific 3' reverse primer flanked by an XbaI site (5'-TCACAGTTTCACAAACCCCAGG-3'). The amplified fragment was cut with EcoRI/XbaI and cloned into a modified version of pCRIII (Invitrogen), in frame with an N-terminal Flag peptide (15). The soluble form of APRIL (sAPRIL) was generated using the two primers (5'-AAACAGAAGAAGCAGCACTCTG-3') and (5'-TCACAGTTTCACAAACCCCAGG-3') containing a PstI and XbaI site, respectively, and subsequently cloned into a modified pCRIII vector, containing both a HA signal for protein secretion in eukaryotic cells and an N-terminal Flag epitope (15).

Example 2

The widespread expression of APRIL in tumor cells and tissues suggested to us that APRIL may be associated with tumor growth, and we therefore incubated various tumor cell lines with purified recombinant Flag-tagged sAPRIL (10).

Human embryonic 293T cells, human leukemia Jurkat T-cells, human Burkitt lymphoma B-cells Raji and melanoma cell lines were grown as previously described (16, 17). Other cell lines referred in this paper are deposited in and described by the American Type Culture Collection (Rockville, Maryland). All cell lines were cultured in RPMI or DMEM medium supplemented with 10% fetal calf serum.

Flag-tagged versions of the extracellular domain (residues 103-281) of human FasL and TRAIL (residues 95-281) were recently described (15). Flag-tagged soluble human TWEAK (residues 141-284) was produced in 293 cells (P. S. manuscript in preparation). The anti-Flag antibody M2 were obtained from Kodak International Biotechnologies. An increase in proliferation of the Jurkat T lymphoma cells in the presence of APRIL was observed in a dose dependent manner as detected by an increase in number (approximately 50%) (11) of viable cells 24 hrs after ligand

addition (Fig. 3A). The proliferation of cells was detemined by incubating cells at 50,000 cells per well in 100 µl medium with the indicated concentrations of recombinant APRIL, TWEAK, TRAIL, FasL and by determining the number of viable cells using the Celltiter 96 AQ proliferation assay (Promega) after 24 hrs, following the manufacturer's instructions, or by ^{3}H-thymidine incorporation. For the immunodepletion of Flag-APRIL, anti-Flag coupled to agarose was used.

The increase in proliferation was independent of a co-stimulatory signals such as anti-CD3 antibodies or other cytokines. As expected, the addition of identically produced and purified FasL to Jurkat cells decreased the number of viable cells, whereas TWEAK had no effect. The increased cell number correlated with augmented (40%) ^{3}H-thymidine incorporation in APRIL-treated cells (Fig. 3A). Immunodepletion of FLAG-tagged APRIL-containing conditioned medium by anti-FLAG antibodies, but not anti-myc antibodies, reduced the proliferative effect (Fig. 3B), indicating that the proliferative effect was specific and due to APRIL. Increased proliferation rates were also seen in some B lymphomas (human Raji, mouse A20 cells, but not human BJAB) and on cell lines of epithelial origin such as COS and HeLa, as well as melanomas (Fig. 3C). The breast carcinoma cell MCF-7 did not respond. The effect on Jurkat cells was even more pronounced when the fetal calf serum was reduced from 10% to 1% (Fig. 3D).

Recombinant sAPRIL has forms aggregates which may explain the rather high concentrations needed to detect a proliferative effect with sAPRIL. We therefore transfected NIH-3T3 cells with full-length human APRIL (12) and obtained several APRIL-expressing clones (Fig. 4A). NIH-3T3 APRIL clones were established using the calcium phosphate method of transfection and the full-length FLAG-tagged APRIL containing pCRIII expression vector. Cellular proteins of about 2 x 10^{6} cells per lane were electrophoretically separated on a 12% polyacrylamide gel in the presence of SDS under reducing conditions and subsequently transferred to nitrocellulose. Immunoblot analysis of Flag-tagged APRIL was conducted using 5 µg/ml of the rat monoclonal anti-Flag antibody M2 (Kodak International Biotechnologies). First antibodies were detected using affinity purified anti-peroxidase-conjugated donkey anti-mouse antibody (Dianova, Hamburg, Germany) followed by a chemiluminescence reaction using the ECL system (Amersham).

Interestingly, APRIL transfectants proliferated faster than mock-transfectants (Fig. 4B). We reasoned that the APRIL-transfected NIH-3T3 cells might also have a

growth advantage *in vivo*. When wild-type or mock-transfected NIH-3T3 cells were injected into nude mice, small palpable tumors were observed after 5-6 weeks (13). In contrast, two clones of NIH-3T3 cells stably transfected with APRIL both induced tumors after only 3-4 weeks. After 6 weeks, mice had to be killed due to the high tumor burden (Fig. 4C). NIH/3T3 fibroblasts (American Type Culture Collection, Rockville, Maryland) and the various transfectants (1×10^5 cells) were suspended in 50 µl PBS and injected subcutaneously into the flank region of BALB/c nude mice (Harlan, Zeist, Netherland). Tumor size was measured every three days. Mice were age-matched (3 animals per group).

Example 3:

Isolation of a receptor binding to APRIL.

Ligands of the TNF family can be used to identify and clone receptors. With the described APRIL sequences, one could fuse the 5' end of the extracellular domain of APRIL which constitutes the receptor binding sequence to a marker or tagging sequence and then add a leader sequence that will force secretion of APRIL in any of a number of expression systems. One example of this technology is described by Browning et al., (1996) (JBC 271, 8618-8626) where the LT-β ligand was secreted in such a form. The VCAM leader sequence was coupled to a short myc peptide tag followed by the extracellular domain of the LT-β. The VCAM sequence is used to force secretion of the normally membrane bound LT-β molecule. The secreted protein retains a myc tag on the N-terminus which does not impair the ability to bind to a receptor. Such a secreted protein can be expressed in either transiently transfected Cos cells or a similar system, e.g., EBNA derived vectors, insect cell/baculovirus, picchia etc. The unpurified cell supernatant can be used as a source of the tagged ligand.

Cells expressing the receptor can be identified by exposing them to the tagged ligand. Cells with bound ligand are identified in a FACS experiment by labeling the myc tag with an anti-myc peptide antibody (9E10) followed by phycoerythrin (or a similar label) labeled anti-mouse immunoglobulin. FACS positive cells can be readily identified and would serve as a source of RNA encoding for the receptor. An expression library would then be prepared from this RNA via standard techniques and separated into pools. Pools of clones would be transfected into a suitable host cell and binding of the tagged ligand to receptor positive transfected cells determined via

microscopic examination, following labeling of bound myc peptide tag with an enzyme labeled anti-mouse Ig reagent, i.e. galactosidase, alkaline phosphatase or luciferase labeled antibody. Once a positive pool has been identified, the pool size would be reduced until the receptor encoding cDNA is identified. This procedure could be carried out with either the mouse or human APRIL, as one may more readily lead to a receptor.

It will be apparent to those skilled in the art that various modifications and variations can be made in APRIL, compositions and methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

I claim:

1. A DNA sequence encoding APRIL or a fragment thereof.

2. A DNA sequence encoding APRIL said sequence consisting essentially of SEQ. ID. NO.2

3. A DNA sequence consisting essentially of SEQ. ID. NO. 1 said DNA encoding a polypeptide, said polypeptide consisting essentially of SEQ. ID. NO. 2.

4. A DNA sequence that hybridizes to at least a fragment of SEQ. ID NO. 1 said fragment comprising at least 20 consecutive bases, said DNA sequence encoding a polypeptide that is at least 30% homologous with an active site of APRIL.

5. A DNA sequence according to claim 2 wherein said sequence consists essentially of SEQ. ID. NO. 1 with conservative substitutions, alterations or deletions.

6. A recombinant DNA molecule comprising a DNA sequence encoding APRIL said sequence operatively linked to an expression control sequence.

7. The molecule of claim 6 comprising SEQ. ID. NO. 1.

8. A unicellular host transformed with a recombinant DNA molecule of claim 6 or 7.

9. A DNA sequence encoding APRIL having the amino acid sequence of SEQ. ID. NO. 2.

10. A method for producing substantially pure APRIL comprising the step of culturing the unicellular host of claim 8.

11. APRIL essentially free of normally associated animal proteins.

12. The APRIL of claim 11 consisting essentially of SEQ. ID. NO. 2.

13. A pharmaceutical composition comprising a therapeutically effective amount of APRIL or an active fragment thereof, and a pharmaceutically acceptable carrier.

14. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

15. The pharmaceutical composition of claim 13 wherein said APRIL or active fragment thereof comprises SEQ. ID. NO. 2 or a biologically active fragment thereof.

16. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

17. A method for stimulating the immune system comprising administering the composition of claim 13.

18. A method for suppressing the immune system comprising administering an effective amount of the pharmaceutical composition according to claim 13.

19. A method for treating cancer comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 13.

20. A method for identifying a receptor for APRIL comprising:

a. providing APRIL or a fragment thereof,

b. labeling said APRIL or fragment thereof with a detectable label;

c. screening a composition to detect receptors which bind to the detectably labeled of step b.

21. A soluble biologically active fragment of the APRIL of claim 11.

22. A polypeptide comprising an amino acid sequence that is encoded by a DNA selected from the group consisting of:

a. a DNA sequence comprising SEQ. ID. NO. 1;

b. a DNA sequence that hybridizes to the DNA defined in a. and coding on expression for a polypeptide that is at least 40% homologous with the APRIL of claim 12.

23. An antibody preparation that is reactive to APRIL or its receptor or biologically active fragments thereof.

24. The antibody preparation of claim 23 comprising monoclonal antibodies.

25. A method for producing an antibody preparation reactive to APRIL or its receptor comprising the step of immunizing an organism with APRIL or its receptor, or an antigenic fragment thereof.

26. An antisense nucleic acid against APRIL comprising a nucleic acid sequence hybridizing to at least a portion of SEQ. ID. NO. 1.

27. A pharmaceutical composition comprising an antibody preparation according to claim 24.

28. A method of expressing APRIL in a mammalian cell comprising:

a. introducing a gene encoding APRIL into a cell;

b. allowing said cell to live under conditions such that said gene is expressed in said mammal.

29. A method of treating a disorder related to APRIL in a mammal

a. introducing into a cell a therapeutically effective amount of a vector comprising a gene encoding APRIL; and

b. expressing said gene in said mammalian cell.

30. The method of claim 29 wherein the mammal is a human.

31. The method of claim 29 wherein said vector is a virus.

32. A method of inducing cell death comprising the administration of an agent capable of interfering with the binding of APRIL to a receptor.

33. The method of claim 32 further comprising the administration of interferon-γ.

34. A method of treating, suppressing, activating or altering an immune response involving a signaling pathway between APRIL and its receptor, said method comprising the step of administering an effective amount of a blocking agent capable of interfering with the association between APRIL and its receptor.

35. The method of claim 34 wherein said immune response involves human carcinoma cells.

36. A method of treating, suppressing or altering the progression of a cancer comprising adminstering to a patient an effective amount of a blocking agent between April and its receptor capable of interfering with the association.

37. The method of claim 36, wherein the blocking agent is a modified inhibitory form of APRIL, or anti-APRIL antibodies or biologically active fragments thereof.

38. The method of Claim 37 wherein the blocking agent is an anti-APRIL receptor antibody.

39. The method of Claim 36 wherein the blocking agent is administered to a patient in combination with at least one chemotherapeutic agent.

40. The method of claim 39 further comprising the step of administering radiation therapy to said patient.

Fig. 1

A
TM
50
100
150
200
250
IsAPRIL
B
APRIL
TNFa
LT a
FASL
TRAIL
TWEAK
TRANCE

Fig. 2

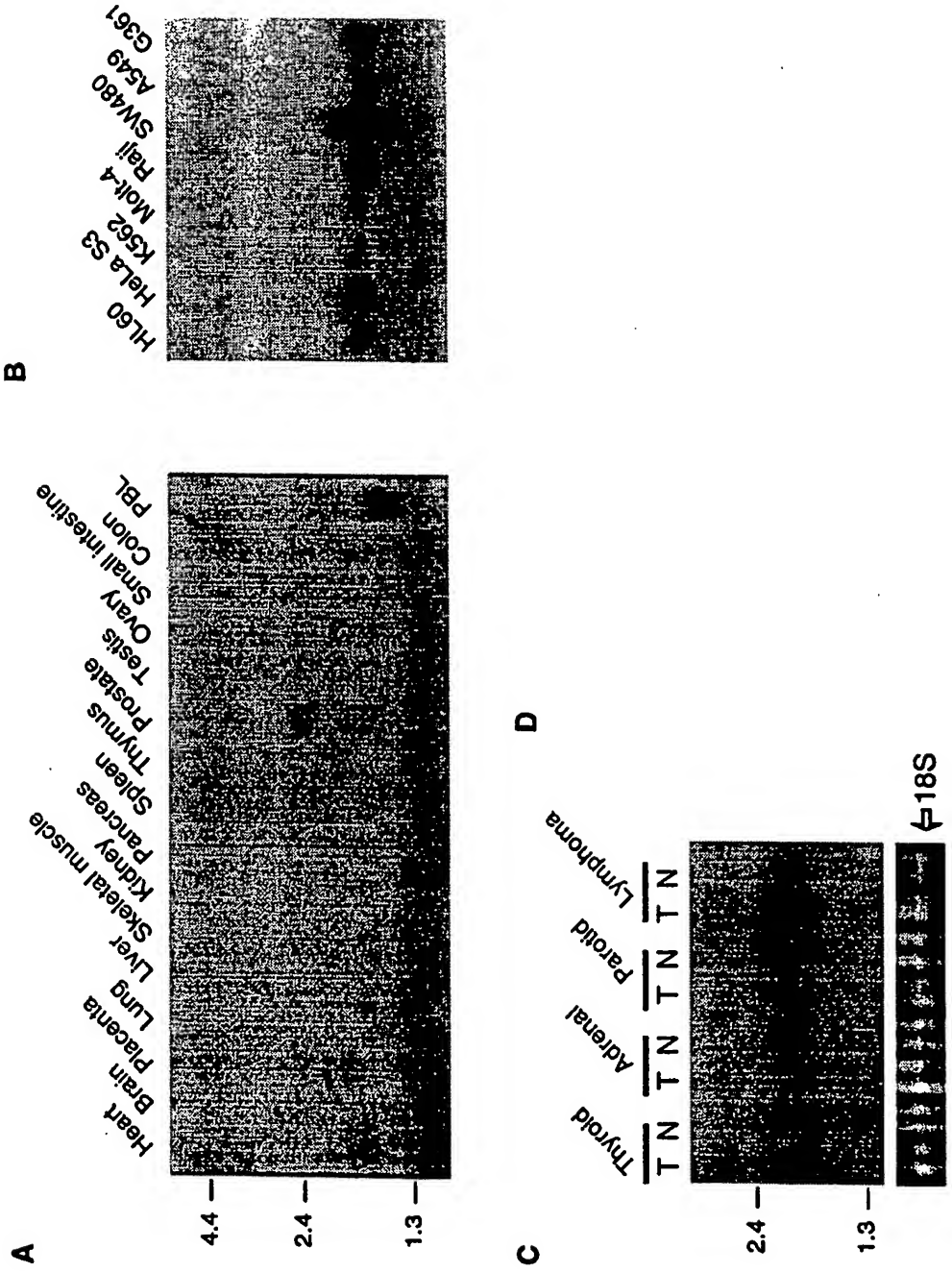
A
Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
PBL
4.4
2.4
1.3
B
HL60
HeLa S3
K562
Molt-4
Raji
SW480
A549
G361
C
T N T N T N T N
Thyroid
Adrenal
Parotid
Lymphoma
2.4
1.3
D
18S

Fig. 2D

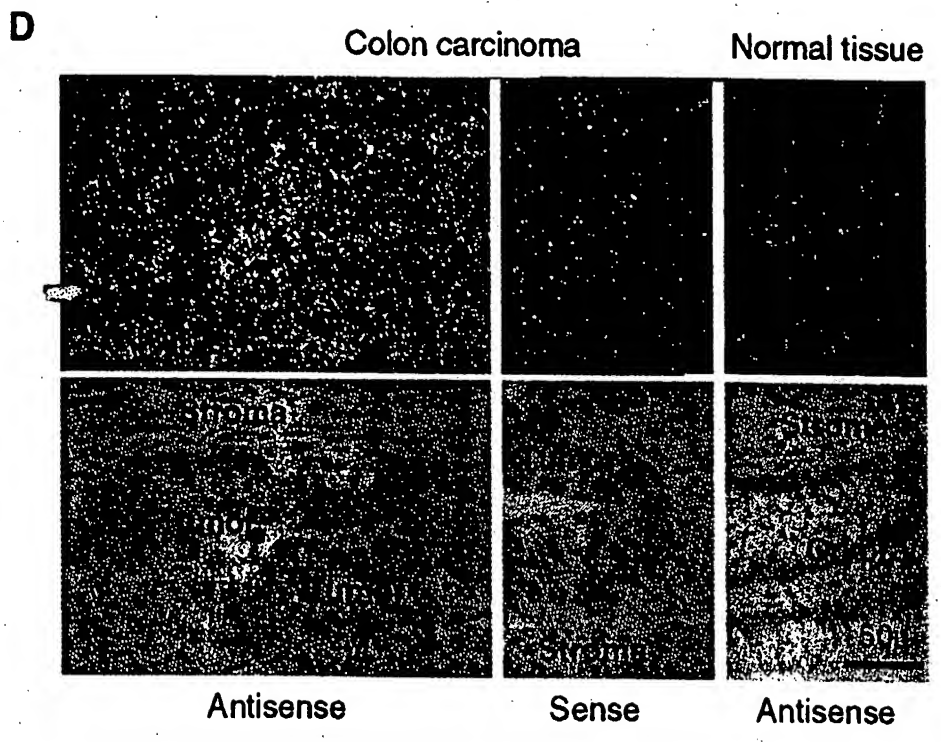
D
Colon carcinoma
Normal tissue
stroma
stroma
stroma
50
Antisense
Sense
Antisense

Fig. 3

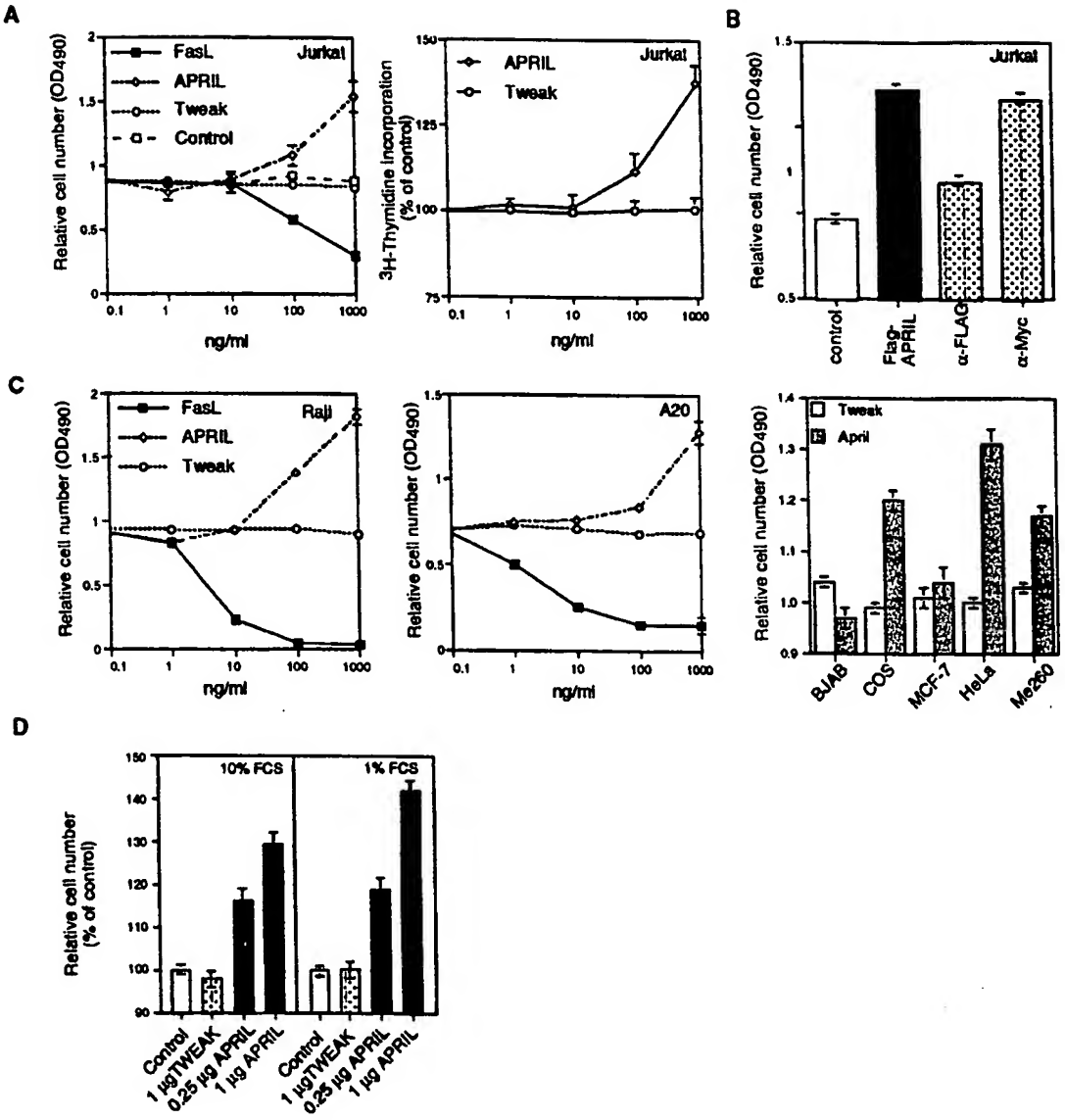
A
Relative cell number (OD490)
FasL
APRIL
Tweak
Control
Jurkat
ng/ml
^{3}H-Thymidine incorporation (% of control)
APRIL
Tweak
Jurkat
ng/ml
B
Relative cell number (OD490)
Jurkat
control
Flag-APRIL
α-FLAG
α-Myc
C
Relative cell number (OD490)
FasL
APRIL
Tweak
Raji
ng/ml
Relative cell number (OD490)
A20
ng/ml
Relative cell number (OD490)
Tweak
April
BJAB
COS
MCF-7
HeLa
Me260
D
Relative cell number (% of control)
10% FCS
1% FCS
Control
1 µgTWEAK
0.25 µg APRIL
1 µg APRIL
Control
1 µgTWEAK
0.25 µg APRIL
1 µg APRIL

Fig. 4

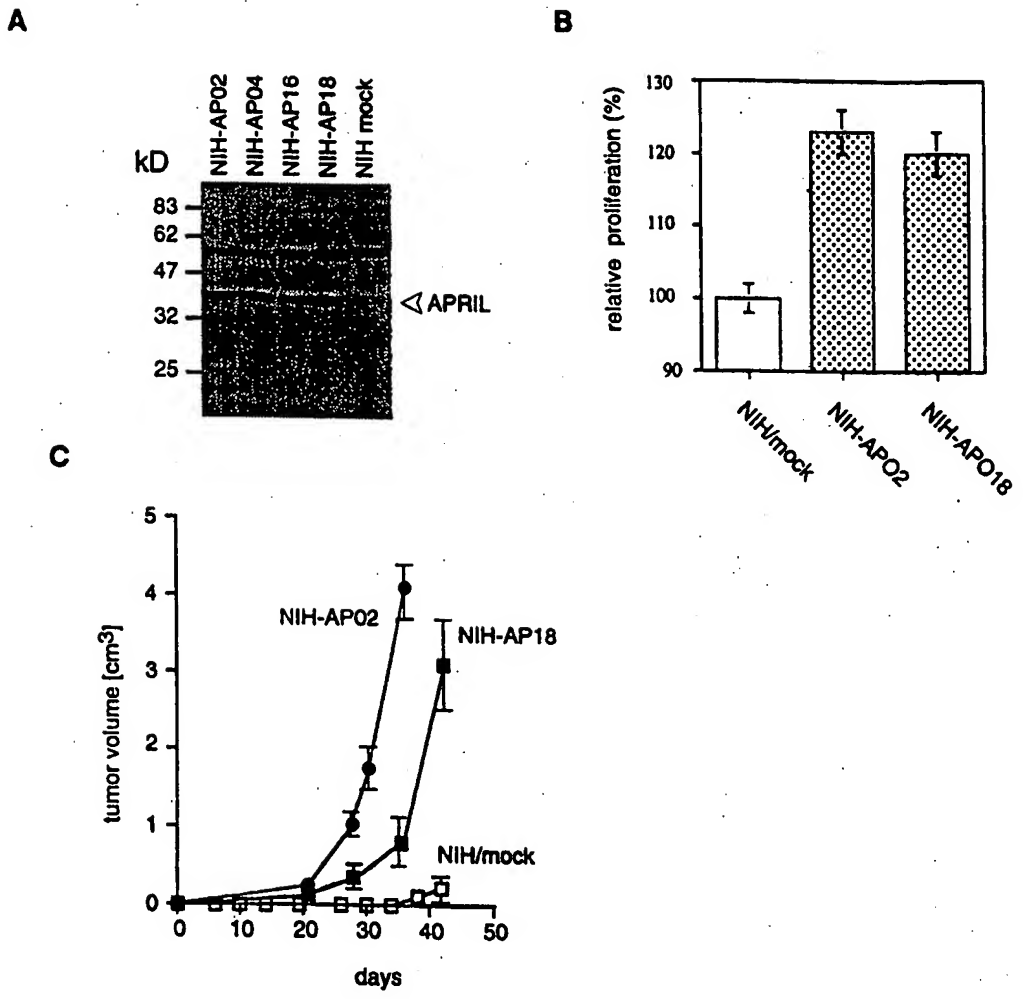
A
NIH-AP02
NIH-AP04
NIH-AP16
NIH-AP18
NIH mock
kD
83
62
47
32
25
APRIL
B
relative proliferation (%)
130
120
110
100
90
NIH/mock
NIH-AP02
NIH-AP18
C
tumor volume [cm3]
5
4
3
2
1
0
NIH-AP02
NIH-AP18
NIH/mock
0
10
20
30
40
50
days

Fig. 5

Comparison Human and Mouse APRIL

```
                                    ... ............. .. .... .. ...
hAPRIL                          MGGPVREPALSVALWLSWGAALGAVACAMALLT
mAPRIL                          MGGSVREPALSVALWLSWGAVLGAVTCAVALLI
                    ...... .......... .....  ..... ... . .... .  .
hAPRIL          QQTELQSLRREVSRLQGTGGPSQNGEGYPWQSLPEQSSDALEAWENGERS
mAPRIL          QQTELQTLRREVSRLQRSGGPSQKQGERPWQSLWEQSPDVLEAWKDGAKS
                    . ........ .. ........ . .... .......... ....... .
hAPRIL          RKRRAVLTQKQKKQHSVLHLVPINATSKDDSDVTEVMWQPALRRGRGLQA
mAPRIL          RRRRAVLTQKHKKKHSVLHLVPVNITSKD-SDVTEVMWQPVLRRGRGLEA
                    . . ..  . . ......... ................. ..........
hAPRIL          QGYGVRIQDAGVYLLYSQVLFQDVTFTMGQVVSREGQGRQETLFRCIRSM
mAPRIL          QGDIVRVWDTGIYLLYSQVLFHDVTFTMGQVVSREGQGRRETLFRCIRSM
                    .. ......................  . .... ... ............
hAPRIL          PSHPDRAYNSCYSAGVFHLHQGDILSVIIPRARAKLNLSPHGTFLGFVKL
mAPRIL          PSDPDRAYNSCYSAGVFHLHQGDIITVKIPRANAKLSLSPHGTFLGFVKL
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SEQ ID NO: 1

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  51 GAGGGTGGAG GGTCTCAAGG CAACGCTGGC CCCACGACGG AGTGCCAGGA
 101 GCACTAACAG TACCCTTAGC TTGCTTTCCT CCTCCCTCCT TTTTATTTTC
 151 AAGTTCCTTT TTATTTCTCC TTGCGTAACA ACCTTCTTCC CTTCTGCACC
 201 ACTGCCCGTA CCCTTACCCG CCCCGCCACC TCCTTGCTAC CCCACTCTTG
 251 AAACCACAGC TGTTGGCAGG GTCCCCAGCT CATGCCAGCC TCATCTCCTT
 301 TCTTGCTAGC CCCCAAAGGG CCTCCAGGCA ACATGGGGGG CCCAGTCAGA
 351 GAGCCGGCAC TCTCAGTTGC CCTCTGGTTG AGTTGGGGGG CAGCTCTGGG
 401 GGCCGTGGCT TGTGCCATGG CTCTGCTGAC CCAACAAACA GAGCTGCAGA
 451 GCCTCAGGAG AGAGGTGAGC CGGCTGCAGG GGACAGGAGG CCCTCCCAG
 501 AATGGGGAAG GGTATCCCTG GCAGAGTCTC CCGGAGCAGA GTTCCGATGC
 551 CCTGGAAGCC TGGGAGAATG GGGAGAGATC CCGGAAAAGG GAGCAGTGC
 601 TCACCCAAAA ACAGAAGAAG CAGCACTCTG TCCTGCACCT GGTTCCCATT
 651 AACGCCACCT CCAAGGATGA CTCCGATGTG ACAGAGGTGA TGTGGCAACC
 701 AGCTCTTAGG CGTGGGAGAG GCCTACAGGC CCAAGGATAT GGTGTCCGAA
 751 TCCAGGATGC TGGAGTTTAT CTGCTGTATA GCCAGGTCCT GTTTCAAGAC
 801 GTGACTTTCA CCATGGGTCA GGTGGTGTCT CGAGAAGGCC AAGGAAGGCA
 851 GGAGACTCTA TTCCGATGTA TAAGAAGTAT GCCCTCCCAC CCGGACCGGG
 901 CCTACAACAG CTGCTATAGC GCAGGTGTCT TCCATTTACA CCAAGGGGAT
 951 ATTCTGAGTG TCATAATTCC CCGGGCAAGG GCGAAACTTA ACCTCTCTCC
1001 ACATGGAACC TTCCTGGGGT TTGTGAAACT GTGATTGTGT TATAAAAAGT
1051 GGCTCCCAGC TTGGAAGACC AGGGTGGGTA CATACTGGAGACAGCCAAGA
1101 GCTGAGTATA TAAAGGAGAG GGAATGTGCA GGAACAGAGGCATCTTCCTG
1151 GGTTTGGCTC CCCGTTCCTC ACTTTTCCCT TTTCATTCCC ACCCCCTAGA
1201 CTTTGATTTT ACGGATATCT TGCTTCTGTT CCCCATGGAG CTCCGAATTC
1251 TTGCGTGTGT GTAGATGAGG GGCGGGGGAC GGGCGCCAGG CATTGTTCAG
1301 ACCTGGTCGG GGCCCACTGG AAGCATCCAG AACAGCACCA CCATCTTA
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SEQ ID NO: 2

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   1 MPASSPFLLA PKGPPGNMGG PVREPALSVA LWLSWGAALG AVACAMALLT
  51 QQTELQSLRR EVSRLQGTGG PSQNGEGYPW QSLPEQSSDA LEAWENGERS
 101 RKRRAVLTQK QKKQHSVLHL VPINATSKDD SDVTEVMWQP ALRRGRGLQA
 151 QGYGVRIQDA GVYLLYSQVL FQDVTFTMGQ VVSREGQGRQ ETLFRCIRSM
 201 PSHPDRAYNS CYSAGVFHLH QGDILSVIIP RARAKLNLSP HGTFLGFVKL
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SEQ ID NO: 3

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   1 GAATTCGGCA CGAGGCTCCA GGCCACATGG GGGGCTCAGT CAGAGAGCCA
  51 GCCCTTTCGG TTGCTCTTTG GTTGAGTTGG GGGGCAGTTC TGGGGGCTGT
 101 GACTTGTGCT GTCGCACTAC TGATCCAACA GACAGAGCTG CAAAGCCTAA
 151 GGCGGGAGGT GAGCCGGCTG CAGCGGAGTG GAGGGCCTTC CCAGAAGCAG
 201 GGAGAGCGCC CATGGCAGAG CCTCTGGGAG CAGAGTCCTG ATGTCCTGGA
 251 AGCCTGGAAG GATGGGGCGA AATCTCGGAG AAGGAGAGCA GTACTCACCC
 301 AGAAGCACAA GAAGAAGCAC TCAGTCCTGC ATCTTGTTCC AGTTAACATT
 351 ACCTCCAAGG ACTCTGACGT GACAGAGGTG ATGTGGCAAC CAGTACTTAG
 401 GCGTGGGAGA GGCCCTGGAG GCCCAGGGAG ACATTGTACG AGTCTGGGAC
 451 ACTGGAATTT ATCTGCTCTA TAGTCAGGTC CTGTTTCATG ATGTGACTTT
 501 CACAATGGGT CAGGTGGTAT CTCGGGAAGG ACAAGGGAGA AGAGAAACTC
 551 TATTCCGATG TATCAGAAGT ATGCCTTCTG ATCCTGACCG TGCCTACAAT
 601 AGCTGCTACA GTGCAGGTGT CTTTCATTTA CATCAAGGGG ATATTATCAC
 651 TGTCAAAATT CCACGGGCAA ACGCAAAACT TAGCCTTTCT CCGCATGGAA
 701 CATTCCTGGG GTTTGTGAAA CTATGATTGT TATAAAGGGG GTGGGGATTT
 751 CCCATTCCAA AAACTGGCTA GACAAAGGAC AAGGAACGGT CAAGAACAGC
 801 TCTCCATGGC TTTGCCTTGA CTGTTGTTCC TCCCTTTGCC TTTCCCGCTC
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SEQ ID NO: 4

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     51  SGGPSQKQGE RPWQSLWEQS PDVLEAWKDG AKSRRRRAVL TQKHKKKHSV
    101  LHLVPVNITS KDSDVTEVMW QPVLRRGRGP GGQGDIVRVW DTGIYLLYSQ
    151  VLFHDVTFTM GQVVSREGQG RRETLFRCIR SMPSDPDRAY NSCYSAGVFH
    201  LHQGDIITVK IPRANAKLSL SPHGTFLGFV KL
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